DEEL 13 -15 1947 -49

ANTONIE VAN LEEUWENHOEK

Journal of Microbiology and Serology

DELPHIS NATVS MDCXXXII. ANTONIVS LEEUWENHOEKIVS

Official publication of the

„NEDERLANDSCHE VEREENIGING VOOR MICROBIOLOGIE"
(Netherlands Society of Microbiology)

Board of Editors: J. P. Bijl, Leiden; H. W. Julius, Utrecht;
A. J. Kluyver, Delft; C. F. van Oyen, Utrecht;
A. Charl. Ruys, Amsterdam; Jan Smit, Wageningen;
E. P. Snijders, Amsterdam; Joh^{A}. Westerdijk, Baarn.

Editors:
MARIE P. LÖHNIS, Wageningen.
L. H. C. PERQUIN, Breda.

SWETS EN ZEITLINGER
BOEKHANDEL EN UITGEVERSMAATSCHAPPIJ, AMSTERDAM

All rights reserved

65-13517

CONTENTS TABLE DES MATIÈRES INHALT

Page

Abdoelrachman, R., Comparative investigations into the influence of the presence of bacteria on the life of pathogenic and apathogenic Leptospirae. 21

Bekker, J. H., Studies on Staphylococci. I. The plasma coagulation reaction. 63

——, Studies on Staphylococci. II. Antistaphylocoagulase in human serum 128

Bosgra, O., The sealing under vacuum in ampoules of freeze dried suspensions of bacteria and viruses 165

Clarenburg, A., Dröes, P. C. and Ruys, A. Charlotte, A case of acute gastro-enteritis caused by *S. manhatten* . 162

Dröes, P. C. s. Clarenburg, A.

Galesloot, Th. E., The cause of the early gas defect in cheese and the means to prevent it 67

Genderen, H. van and Scholtens, R. Th., A photometer for the extinctiometric determination of the density of suspensions of microorganisms with minimal deviation from the law of Beer-Lambert. 153

Giesberger, G., Some observations on the culture, physiology and morphology of some brown-red *Rhodospirillum*-species 135

Kluyver, A. J., Three decades progress in Microbiology . . 1

Kneteman, A., A reaction on the formation of hydrogen peroxide by microorganisms in solid media 55

Otten, L., Investigations into Rabies II 101

Quispel, A., The influence of the oxidation-reduction potential of the medium upon the growth of *Azotobacter chroococcum* 33

Ruys, A. Charlotte s. Clarenburg, A.

Scholtens, R. Th. s. Genderen, H. van

Terpstra, J. I., A peculiar example of bacterial antagonism 44

Verlinde, J. D., Virus infection of the bone-marrow . . . 149

Abstracts 96, 170

(From the Laboratory of Microbiology, Technical University, Delft).

THREE DECADES PROGRESS IN MICROBIOLOGY ¹)

by

A. J. KLUYVER

Lecture held on May 8th 1946 for „Danmarks Naturvidenskabelige Samfund" and the „Biologisk Selskab" after the „EMIL CHR. HANSEN Medal" had been conferred on him,

(Received March 15, 1947).

It is only appropriate that our first thoughts this afternoon go out to that pioneer in the field of microbiology whose birthday, now 104 years ago, we commemorate today. In these surroundings it may be deemed superfluous to pause upon the many important contributions EMIL CHR. HANSEN has made to pure and applied microbiology during his well-used life-time. There is, however, every reason for the lecturer to acknowledge his great debt of gratitude towards the man who in his last will took measures to promote even after his death the development of his beloved science for long times to come. In saying this I do not wish to suggest that the superficial considerations which I have the honour to present before you this afternoon will influence in the least the growth of microbiology. But I want to give expression to my conviction that owing to the contact with so many distinguished representatives of Danish science I shall be on my returning home, if not a better man, at least a better scientist. And it is also at this international diffusion of knowledge that HANSEN must have aimed, when he decided to call into existence the „Fond" which bears his name.

Having gone back in our minds to the year 1909, incidentally the year in which my own initiation in the world of microbes took place, it is tempting to take a retrospective view of the development of microbiology as a science, and to compare the present situation with the stage reached about thirty years ago. Such a survey can, of course, not aim at completeness, and I shall have to confine myself to a passing into review some of the major trends of investigation which on looking backward come to the fore. In doing so I hope to bring into relief the considerable progress made in three decades of almost restless research, a progress which is characterized by an ever-increasing recognition of the unity that is at the basis of the at first sight so bewildering diversity in the various manifestations of microbe life.

---

¹) At the special request of the Editors prof. KLUYVER consented to the reprint of his lecture.

Let us now first dwell for a moment on the stage of development microbiology had reached in the period immediately after HANSEN's decease.

Seen from the distance of to-day it is clear that microbiological science passed through a phase of transition then. The glorious period of exploration of the microbe world had practically come to an end. The preceding quarter of a century had witnessed an almost endless series of triumphs in the discovery and isolation of numerous microbial species. In the hands of masters like WINOGRADSKY and BEIJERINCK the principle of the elective culture had opened the world of invisible life. Owing to the so pronounced diversity in nutritional requirements of the various types of micro-organisms it proved possible to bring about accumulations of many diverse microbes, thus facilitating their isolation and a first investigation of their properties. By these studies a clear light was thrown on the almost ubiquitous occurrence of numerous microbes on earth. But still more impressive was the manifestation of the extreme diversity of chemical systems which proved to be able to support microbial life. I have only to remind you of WINOGRADSKY's great discovery of the so-called autotrophic bacteria characterized by their ability to proliferate in fully inorganic media in the absence of radiant energy. Other forms of life again were found which satisfied their nutritional demands with single organic compounds either considered to be almost chemically inert like the paraffin hydrocarbons, or even known to be harmful for the greater part of microbes like phenols. In short it can be said that one specialist amongst the microbes was hardly discovered, or already a second specialist with still greater achievements was being announced. The microbiological scenery seemed to be one grand scientific ,,Tivoli".

However, around 1910 the activity in this direction had obviously slackened: the great explorers of the microbe world had done their job efficiently. One would go too far by stating that since then no new species have been discovered. But how poor has the harvest been in later years when compared with that in the earlier period. And this notwithstanding the enormous increase in the number of ,,microbe-hunters" during the last three decades. Is it not characteristic that the almost incredible extension of the study of bacteriology in the ,,New World" since the first world war has not at all led to the discovery of a ,,new microbe world"? It is true that the number of micro-organisms annually described as new species is still alarmingly large. But how many of these species can rightly be claimed as such, and if so cannot they nearly always be connected with other well-known forms, from which they only differ in secondary characters?

It will be clear that the conclusion of the exploration period in microbiology did not put an end to the growth of this science. It only meant that new trends of investigation manifested themselves, all tending to consolidate the occupied territory by gaining a deeper insight into the properties of the newly discovered world. Evidently a first necessity hereto was to replace the chaotical conglomerate of known living forms by a more or less systematic arrangement. In first instance this asked for a recognition and evaluation of natural relationships between the various forms, and the demarcation of natural groups as building stones for a satisfactory classification.

If for a moment we restrict ourselves to the bacteria, we have to acknowledge that the older workers too had already made several efforts to arrive at some system of classification. But these systems all had the limitation that they were based solely on the scanty morphological data derived from the microscopical examination of the organisms in question.

It is now the great merit of the nestor of Danish bacteriologists, ORLA-JENSEN, to have introduced a new era in the study of bacteriological classification. In several papers JENSEN has made an eloquent and broadly documented plea that physiological characters should determine the main lines of a bacterial system. In their entirety JENSEN's views have not been accepted, and yet they mark a milestone in the development of bacterial taxonomy. For they have opened the eyes of generations of bacteriologists to the truth that, given the enormous diversity of metabolic properties amongst bacteria which contrasts so sharply with the relatively great uniformity characteristic of the metabolism of higher plants and animals, it is not only permissible, but even imperative also to take the physiological properties into account in the ordering of the bacterial kingdom. For those bacteriologists who have not yet been reconciled with this idea it may be remarked that nowadays there is increasing evidence that differences in catabolic properties of bacterial cells can be considered as expressions of variations in submicroscopical morphology. Anyhow, there is no doubt that ORLA-JENSEN's views have greatly influenced the numerous proposals for bacterial classification put forward after 1910. It is true that we still are far from an in all respects satisfactory system, nor can we hope that such an aim will ever be attained. But nevertheless, it can be stated that the phylogenetic approach to the solution of this problem which STANIER and VAN NIEL have brought in their recent study, has thrown a clear light on the interrelationships of the main bacterial groups, and their place in the natural kingdom. And it seems likely that a future more detailed system will be based on the same judicious balance in the evaluation of both morphological and physiological properties which is characteristic of the study of the American authors.

A second feature of the tendency to bring order into the world of microbes has been the foundation of several institutions where collections of living micro-organisms have been brought together and are maintained. As such mention may be made of the ,,Centraalbureau'' for cultures of fungi, including yeasts, founded already in 1906 in my own country, the National Collection of Type Cultures established at the Lister Institute in London, the American Type

Culture Collection formerly in Chicago, now in Washington, and the new, economically very important collections of moulds and yeasts established in the Northern Regional Research Laboratory at Peoria (Ill.). Moreover, several scientists who have been specializing on certain groups have maintained private collections, and it should be mentioned here that investigators in demand of cultures of lactic acid bacteria will seldom have appealed in vain to ORLA-JENSEN's generous cooperation.

It seems certain that collections like those mentioned serve several useful purposes. In the first place they promote the dissemination of knowledge by providing scientists all over the world with authentic cultures of organisms discovered or described by their colleagues elsewhere. Secondly these institutions often develop into centres where special groups of organisms are more or less exhaustively studied from both a systematic and a physiological standpoint. This not rarely leads to the publication of monographs dealing with such groups, of which I need only mention ORLA-JENSEN's two volumes dealing with ,,The Lactic Acid Bacteria'' in order to impress the importance of such studies upon you.

Looking back once more into the post-explorative era of microbiology other trends of investigation besides that of ordering and classification are easily discerned.

In the foregoing I have already emphasized the diversity in metabolism characteristic for the various types of micro-organisms, and it is only self-evident that the often quite amazing chemical activities of many microbes has led to attempts to penetrate deeper into the secrets of their metabolism. In order to give you some idea of the achievements attained along this line during the last three decades I should remind you that in metabolism two counterparts can and should be distinguished, *viz.*, anabolism and catabolism. The term anabolism comprises the processes leading to the building up of cell constituents, while we designate with catabolism those processes which are only of significance to the cell from the standpoint of energy supply.

As for the catabolic processes in higher organisms, plants as well as animals, it has been known since LAVOISIER's fundamental observations that they have to be considered as a slow combustion process of the carbohydrate constituents of the food, and that, therefore, they are characterized by a consumption of oxygen and a simultaneous production of an equivalent amount of carbon dioxide. This so-called respiration process has consequently long been considered to be an essential attribute of life.

Already a first survey of microbial life did bring to light that herein this uniformity in catabolism so characteristic of the higher forms of life is absent. In the first place it soon became evident that, although the proliferation of many microbes is indeed dependent on the availability of free oxygen, the respiration processes differed widely in the nature of the compounds acceptable as substrates for the oxidation process. Moreover, with many types of microbes this process did not yield the final oxidation products carbon dioxide and water, but led to the excretion of incomplete oxidation products in the medium.

Even more startling was the observation made by PASTEUR already in 1860 that there were several microbes which not only could thrive in the absence of oxygen, but for which this gas even was decisively harmful. It did, however, not escape PASTEUR'S attention that this unexpected observation correlated with the occurrence of other chemical conversions known from time immemorial as fermentation (or putrefaction), and his genius made him at once conclude to the physiological equivalence of respiration and fermentation.

Now it gradually became evident that the fermentation processes brought about by various types of micro-organisms mutually show great differences. Although they may attack the same substrate, the products of the conversion often differ considerably. While in the best-known fermentation process, the conversion of sugar into carbon dioxide and ethyl alcohol, the products are stoechiometrically related both mutually and to the sugar fermented, in many other cases in which numerous fermentation products are formed such relations do not exist. Moreover, the quantities in which these products are formed may vary largely owing to differences in the external conditions to which the fermenting organism is exposed.

Altogether, a quarter of a century ago the biochemistry of catabolic processes in the microbe world showed such an extreme diversity that at that time one might well despair of any attempt to elucidate this superchemistry.

It is undoubtedly one of the greatest results of three decades of microbiological investigation that nowadays this aspect has so fundamentally changed. If we try to survey the causes which have led to this revolution it is at once clear that we owe this progress mainly to the splendid investigations of both MEYERHOF and WARBURG and their co-workers. Concentrating their efforts on an analysis of the processes of alcoholic fermentation and glycolysis, and building on the work of pioneers like HARDEN and NEUBERG, they have been able to give us a clear insight into these processes.

Stimulated by these so successful investigations other types of fermentations have likewise been more or less intensively studied in several other laboratories. The chief result of all this has been that the so amazing diversity of the various fermentation processes has given way to a quite unexpected unity in principle.

The main points of the insight gained can be summarized as follows.

All these fermentation processes consist of a series of individual step-reactions, each of which constitutes a chemically intelligible, and extremely simple type of reaction, the greater part of these step-reactions being typical equilibrium reactions. Moreover they show great uniformity in so far that many of them can be characterized as reactions in which hydrogen atoms (or electrons) are transferred from one constituent to another.

Another feature of our present insight into the fermentation processes is, however, the important rôle which phosphoric esters play therein. Not only must we accept that the first conversion of the carbohydrate to be fermented is the formation of a hexosephosphoric acid, but it has also become increasingly clear that a transference of the phosphate group from one intermediate product to a second one, ultimately leading to a closed phosphate cycle, is an essential counterpart of the hydrogen transference just mentioned. The more so, because at least in several cases a direct linkage between the two types of processes has been proved to exist.

Especially gratifying is the result that in all these fermentation processes the first steps of the breakdown of the carbohydrate have been proved to be the same, and that only in the final stages of this breakdown process specific step-reactions take place which are responsible for the differences in the final fermentation products.

Still more important is that these attempts to arrive at an unification of microbial catabolism have not remained restricted to the typical cases of sugar fermentation. In this connection I mention only that also for the long known bacterial processes of denitrification, sulphate reduction and methane fermentation it has been shown that they represent typical cases of catalytic hydrogen transference in which respectively nitrates, sulphates and carbonates act as hydrogen acceptors.

The greatest triumph of this analysis of microbial catabolism is, however, that it has proved possible to extend these unifying efforts also to the various types of respiration processes encountered in the microbe world. Numerous investigations initiated by WIELAND's classical observations leave no doubt that these processes too consist of a series of step-reactions of the hydrogen or electron transference type, the free oxygen acting as the final hydrogen acceptor. This, of course, is a result with wide implications. For it means that at the bottom of the physiological equivalence of respiration and fermentation as advocated by PASTEUR there is a conformity in essence which is highly edifying to the scientific mind. It is clear that hereby new perspectives have been opened for a satisfactory understanding of the interrelation between fermentation and respiration in one and the same type of cell as manifested in the so-called reaction of PASTEUR. But the unification in question also opens the way for a better appreciation of evolutionary developments which have taken place in the microbe world, since the antithesis between the aerobic and anaerobic mode of life has been largely removed.

The question now arises in how far three decades of biochemical investigations which have added so much to our insight into microbial catabolism have also succeeded in elucidating the anabolic counterpart of metabolism. Even a superficial survey of anabolism suffices to recognize that the task of the biochemist in this respect is tremendous. The building up of the thousands of chemically widely differing cell constituents starting from the compounds of the food is especially in the microbe world an extremely impressive problem, for numerous are the instances in which microbes are performing this miracle in media in which the number of the organic constituents is restricted to one or two. This obviously means that proliferation of a micro-organism in such a medium involves a super-chemistry far beyond the imagination of the boldest and cleverest of our organic chemists.

Nevertheless we may state that in this domain too real progress has been made in the period under consideration. Much of this progress is closely linked up with the advances made regarding our knowledge of catabolic processes. This becomes at once clear when we first consider what may be called the main aspect of anabolism.

Thirty years ago the view was generally held that in anabolism we are dealing with a very special type of chemistry. For it is of common experience that in these anabolic processes often compounds are formed with an energy content which greatly surpasses that of the substrate of the synthesis. I need only remind you of the formation of fat by cells which develop in a medium with sugars as a sole source of carbon. Hence it was concluded that in anabolic processes we were dealing with reactions of a very special kind unknown in ordinary chemistry, so-called involuntary reactions, in which the free energy of the system increases. This was deemed possible because these reactions acquire energy from the simultaneously occurring catabolic processes in which the free energy decreases, and this to such an extent that the free energy of the complex system as a whole decreases as well.

It is easily understood that gradually much opposition has developed against this conception of an energetic coupling of two chemical reactions which do not have any material link in common. It is, therefore, most rejoicing that in the last ten years considerations have been put forward which seem suitable to free biochemistry from this special vitalistic aspect.

I have already remarked that the greater part of the step-reactions in the catabolic breakdown of carbohydrates are of the equilibrium type. This implies that the addition of many of the intermediate products to the cell must automatically lead to a partial reversal of the breakdown process, *i.e.* to the synthesis of the carbohydrate. The realisation of this situation made CORI succeed in the long-pursued enzymatic synthesis of glycogen and starch starting from glucose-1-phosphate or CORI-ester. Perhaps still more spectacular has been the successful solution found by DOUDOROFF and his co-workers for the problem of the synthesis of saccharose, a problem which generations of organic chemists have been unable to solve. With the aid of a phosphorylase preparation obtained from some common bacterium saccharose was readily produced from a mixture of CORI-ester and fructose.

Now these examples of the successful demonstration of the formation of typical assimilatory products like glycogen and saccharose may perhaps not be so very impressive, because of the relative complexity of the substrate — Cori-ester — from which these syntheses start. But there is no doubt that by applying the necessary biocatalysts they could as successfully be accomplished when starting with various much simpler compounds, such as for instance phosphopyruvate.

Since, however, in these cases the potentials of substrates and synthetical products are very much on the same level they do not represent clear cases of energy transfer necessary for the formation of anabolic products of high potential as referred to earlier.

Now it is very satisfactory that also in the elucidation of the mechanism of such anabolic reactions important advances have been made. These advances are for a great deal due to three scientists well-known in Copenhagen so that it will suffice to give only a very brief review of their findings. The development of our insight into this matter has started with Lundsgaard's discovery of the anaerobic phosphocreatine breakdown during alactacid muscle contraction, and the coupling of the resynthesis of this compound with lactic acid formation in glycolysis. A second well-defined instance of the coupling of an oxidation with phosphate uptake was discovered by Meyerhof and his co-workers in the oxidation of phosphotriose as a step-reaction in carbohydrate breakdown. They showed that this oxidation led to the formation of phosphoglycerylphosphate (diphosphoglyceric acid). The important aspect of this observation is that this oxidation is reversible while the analogous oxidation of the phosphotriose without phosphate is strongly exergonic. We may conclude, therefore, that the diphosphoglyceric acid, owing to the energy-rich acylphosphate bond acts as a storage for the potential energy of the oxidation reaction. Both Lipmann and Kalckar have in recent years discovered several other labile compounds with such energy-rich phosphate bonds, and have dealt in theoretically very important studies with their remarkable properties. There seems to be little doubt that these highly reactive compounds with high energy potential which are formed in catabolic processes act as a starting point for synthetical processes which then again consist of chains of ordinary step-reactions, *i.e.* reactions which proceed with a decrease of free energy.

A striking example in favour of this view may not pass unmentioned. I have already referred to the remarkable metabolism of the so-called chemo-autotrophic bacteria who are able to build up their cell constituents from carbon dioxide owing to the energy derived from inorganic oxidation processes. Until shortly the nature of this energy transfer was still wholly obscure. Vogler, Umbreit and Le Page have now given convincing proof that for the sulphur oxidizing bacterium *Thiobacillus thiooxidans* the formation of organic phosphate compounds provides a material link between catabolism and anabolism. The first advance made by these investigators was the demonstration of the possibility to separate both processes in time. A suspension of the bacteria supplied with a small quantity of sulphur in the absence of carbon dioxide was found to consume oxygen rapidly, and if then after some time carbon dioxide was admitted a rapid uptake of this gas was observed. But the most remarkable fact was that the oxidation of the sulphur proved to be accompanied by the uptake of inorganic phosphate from the medium, whilst in the phase of carbon dioxide assimilation the phosphate was again released. Finally it could be shown that the cells contained several organic phosphate compounds well-known as metabolites in heterotrophic organisms. The intermediate organic phosphate compound which evidently acts as a material link between the energy yielding oxidation and the energy demanding synthesis is to all probability adenosine-3-triphosphate a compound closely related to adenosine-5-triphosphate a regularly occurring intermediate in yeast and muscle metabolism, and which is one of the best-known examples of a compound with an energy-rich phosphate bond.

Here again we are confronted with a most remarkable unity in metabolism of micro-organisms at first sight so widely separated. It is clear that these important results mean a very real contribution to the removal of the contrast between the autotrophic and the heterotrophic mode of life.

It is impossible to discuss thirty years development of our knowledge of microbiological metabolism without giving some special attention to the change in our attitude towards the rôle of carbon dioxide in this metabolism. We can state that until ten years ago the current views were fully governed by the classical picture of the cycle of carbon in nature. According to this picture carbon dioxide had to be considered as the endproduct of respiration in living animals and plants and of their decay after death under the influence of bacteria and fungi. The carbon dioxide once formed was unassailable by all heterotrophic organisms, and only entered into the second half of the cycle owing to the special action of the green plants in which the radiant energy of the sun managed to bring about the reduction ultimately leading to the formation of the various organic constituents of the plant.

Now I shall not enter into details regarding the various observations which are responsible for a considerable change of these views. To begin with I briefly refer to the fact that the discovery of the chemo-autotrophic bacteria already made exception to the rule that the conversion of carbon dioxide to organic compounds could only proceed with the aid of light energy. But besides this exception of a special nature gradually several indications were obtained which seemed to contradict the idea that in the colourless heterotrophic organisms carbon dioxide is only an indifferent endproduct of metabolism. A fast increasing number of instances were recorded all pointing to the fact that small amounts of carbon dioxide are indispensable to the initiation of growth of a great variety of heterotrophic micro-organisms, and that growth may be either completely suppressed or at any rate considerably retarded by a more or less successful removal of carbon dioxide from the culture. This seemed to indicate that the micro-organisms in question in some way or other managed to utilize carbon dioxide for the synthesis of essential cell constituents.

However, apart from the disregarded observation made by Söhngen already in 1905 that methane bacteria are able to convert a mixture of carbon dioxide and hydrogen into methane, it lasted till 1936 before experimental proof for the utilization of carbon dioxide in special cases of heterotrophic metabolism was given. In that year Woods showed that *Bacterium coli* was able to produce formic acid out of carbon dioxide and hydrogen, whilst Barker succeeded in bringing convincing proof that also in the fermentation of organic compounds by methane bacteria the methane always originated from a reduction of carbon dioxide. Now these conversions of carbon dioxide into other compounds with one C-atom were undoubtedly interesting but they did not throw any light on the problem of the indispensability of carbon dioxide for cell synthesis and growth. For this reason it was of great importance that Wood and Werkman, also in 1936, showed that in the fermentation of glycerol the strictly heterotrophic propionic acid bacteria take up carbon dioxide from the medium, and that for every mole of carbon dioxide reduced approximately equivalent quantities of succinic acid with its 4 carbon atoms are formed. This result may be deemed to be of far-reaching significance, since it strongly suggests that here synthesis of a C_4-compound out of a C_3-compound and carbon dioxide has taken place, which would imply the formation of a new bond between carbon atoms. It is easily understood that this experimental result together with the increasing evidence for the indispensability of carbon dioxide for heterotrophic organisms in general made several investigators surmise that the uptake of carbon dioxide into organic compounds under formation of new carbon-carbon bonds would be a reaction of more or less common occurrence in heterotrophic cells.

In 1939, when lecturing on this subject in Helsinki I have expressed this view with the following words: ,,At present a lecturer still must be prepared to meet with energetic protests when he contends that the cattle at pasture, or even his distinguished audience assimilates carbon dioxide. Nevertheless, the temptation to such an assertion is already there!'' Now seven years later the statement in question is no longer open to scepticism, and many of my auditors will think it to be quite commonplace: the synthetic utilisation of carbon dioxide by heterotrophic cells has been indubitably proved for representatives of very much diverging classes of organisms, such as bacteria, yeasts, fungi, protozoa and higher animals.

One might rightly ask for the reasons which are responsible for the fact that such a far-reaching discovery which amongst others noticeably affects our views on the carbon cycle in nature has been made so late in the development of our science. Yet this is easily understood, if one realizes that in all these organisms metabolism is characterized by a net production of carbon dioxide thus rendering it virtually impossible to detect any simultaneous reassimilation of carbon dioxide with the usual chemical or manometrical techniques. It is only the application of a new and powerful tool to the problem, namely the use of „tracer" or „labelled" elements which has made this progress possible.

On the principle of this method which even in the short time of its application has revolutionized several aspects of metabolism I need not digress, since it is again in Copenhagen that it was first introduced into biochemistry by von Hevesy. The remarkable results obtained by this scientist and his followers on introducing compounds containing radioactive phosphorus into metabolic investigation are universally known. But the importance of these studies have perhaps even been surpassed when Schoenheimer and Rittenberg applied the same principle in their researches on nitrogen metabolism by making use of nitrogen compounds containing the stable heavy N^{15} isotope. It now has been the application of radioactive C^{11} carbon by Ruben, Kamen and their co-workers, and of the stable heavy C^{13} isotope by Wood, Werkman and collaborators that has yielded the incontestable proof for the assimilation of carbon dioxide by heterotrophic cells.

However, it should not be inferred that the purport of the recent investigations with the carbon isotopes is restricted to the mere establishment of this carbon dioxide assimilation.

In the first place it has been possible to identify in some instances the primary reactions in which the carbon dioxide is fixed. Thanks to the use of the labelled carbon there is nowadays no longer any doubt that *Bacterium coli* contains enzyme-systems capable of bringing about the carboxylation of pyruvic acid leading to the formation of oxalacetic acid which acid then is converted into succinic, lactic and acetic acids. It was further proved that in accordance with expectation in all these products the labelled carbon was located in the carboxyl group. But more recent studies by Lipmann and Werkman have shown that carboxylation of pyruvic acid is not the only way in which *Bacterium coli* can fix carbon dioxide. The second way is especially interesting because it clearly represents another successful intervention of a compound with an energy-rich phosphate bond. We owe to Lipmann the important observation that the conversion of pyruvic acid by bacterial systems into acetic and formic acid is not, as had long been accepted, a reaction in which water takes part, but a phosphorolysis, in so far that the primary product besides the formic acid is not acetic acid but acetylphosphate. By adding formic acid with labelled carbon to the medium it has been definitely proved that owing to the high potential of the acetylphosphate this primary reaction is reversible, and since *Bacterium coli* is also able to form formic acid out of carbon dioxide and hydrogen it is now quite certain that carbon dioxide can also enter the metabolism via a synthesis of pyruvic acid.

I cannot dismiss the subject of heterotrophic carbon dioxide assimilation without leaving for a moment the microbiological field, and asking your attention for analogous studies made in animal metabolism. Here in some cases it has been possible to trace the path which the labelled carbon follows in the intricate web of metabolic processes. Amongst others this has led to the startling observation that part of the labelled carbon injected intraperitoneally in the form of a sodium bicarbonate solution into fasted rats can within a short time at least partly be found back in the liver glycogen. And Wood and his coworkers have even succeeded in bringing experimental proof that this labelled carbon is located in the positions 3 and 4 of the glucose molecules which are the building stones of the glycogen. This result is so gratifying because it is exactly what might be expected on the basis of the current reaction schemes in the supposition that glycogen synthesis proceeds as the reversal of glycolysis.

Finally it should be observed that in the last three years the use of the stable carbon isotope has found application far beyond the problem of carbon dioxide assimilation. Werkman has succeeded in preparing various acids, such as acetic, lactic, propionic and butyric acids, containing heavy carbon atoms either in the carboxyl groups, or also in other locations in the molecules. By adding such compounds to the usual fermentation media of various types of bacteria they have been able to trace conversions of these molecules which conversions had at least partly a quite unexpected character.

For the moment the number of these observations is still relatively small, but they suffice to show that until shortly the metabolic capacities of the microbial cell have been badly underrated. It now has become increasingly clear that in the metabolizing cell we are dealing with a system of equilibrium reactions in which a continuous interplay between cell constituents, intermediates and so-called endproducts takes place.

This newly gained insight strongly contrasts with the current conception of the preceding period in which fermentation processes, and catabolism in general, were considered more or less as cascades unchained by a more or less stable system of catalysts, and in which a suitable substrate via labile intermediates was transformed into a number of stable endproducts fixed for every type of cell.

It is evident that the introduction of isotopes has brought the study of intermediate metabolism on a level much higher than it had reached before. For it cannot be denied that until recently investigators could not go much farther than to demonstrate that a certain type of cell contained enzyme systems which are capable of bringing about some intermediate step-reaction. But this did not imply that under a given set of metabolic conditions this particular stepreaction does indeed take place. As a matter of fact there have been moments that microbiologists have been struggling with a surplus of intermediate products!

For these reasons it has to be admitted that the drawing up of a scheme for the internal mechanism of a biochemical process on the basis of intermediate products isolated under special conditions has always been afflicted with elements of a speculative character.

If we contrast this situation with the degree of certainty obtainable by the application of labelled atoms which, if properly examined, give straightforward answers to many questions, it is not too dangerous to predict that in the future studies on the intermediate metabolism will almost completely be based on the use of isotopes. Moreover, it does not seem excluded at all that these studies will alter profoundly several of our current views.

There are, however, some considerations which tend to moderate these hopeful expectations, and that is in the first place the at present still very restricted availability of many isotopes, more especially of the carbon isotopes.

In order to apply the radioactive C^{11} carbon successfully it is necessary to work in the immediate neighbourhood of a powerful cyclotron, since the half life time of this isotope is only 21 minutes, restricting its applicability to experiments of a duration of about two hours.

In this respect the stable C^{13} is, of course, quite preferable, but alas the isolation of this isotope obviously still asks for a very cumbersome procedure. This may be inferred from the fact that both WERKMAN and WOOD and their schools have been supplied with C^{13} by the physicist of Minneapolis Professor NIER and that this scientist recently stated that he holds the world record for C^{13}-production amounting to 5 grams annually! And when reading the hopeful statements in scientific journals that this year the Sun Oil Corporation of Philadelphia will start the commercial production of C^{13} it is not generally realized that the annual production by this firm is estimated to be 500 grams! With these facts before our eyes it will be wise to be not too optimistic regarding a world-wide application of carbon isotopes in a near future.

In addition also the following should not be neglected. Publications on metabolic studies with the aid of the stable carbon isotope are as a rule very attractive for the reader, the results reported usually being simple and clear-cut. There is, however, a great danger that one will be insufficiently impressed by the enormous amount of experimental work which has been required in order to obtain these results. In many cases it has been necessary to search a great number of compounds on a possible excess content of labelled carbon, with only a restricted number of positive results. Especially when an attempt is made to arrive at a complete balance of labelled carbon added and recovered many very careful analyses with the mass-spectrometer are indispensable. And if finally it becomes necessary to fix the location of the heavy carbon atoms in the molecule of a metabolic product, various purely chemical degradation reactions of this product have to be studied before the mass-spectometer can again give the desired answer. To this should be added the necessity of carefully recovering in all this work the precious isotope!

All this asks for a more or less specialized staff of workers and, therefore, tends to limit the immediate universal introduction of the isotope method in the study of metabolism. It is, therefore most welcome that in the last years in the United States quite another line of attack in the study of metabolic processes has been discovered which seems to be full of promise and to which I shall return later on.

A survey of thirty years progress in microbiological metabolism would be very incomplete, if not due attention was given to the enormous amount of very successful work which has been performed on the subject of growth factors, leading to the discovery of the rather common occurrence amongst micro-organisms of a phenomenon that later has been termed auxo-heterotrophy by SCHOPFER. As is well-known the first observations regarding this phenomenon date back to 1903 when WILDIERS demonstrated that a certain strain of yeast would only proliferate in a medium containing sugar and the necessary quantities of inorganic nutrilites, if a small quantity of yeast extract or some other vegetable extract was added to the medium. For the growth-promoting principle present in these extracts WILDIERS coined the term bios. The development in this field is too generally known to be in need of further documentation, yet it seems worth while to devote a few remarks to the general aspects which have resulted from these studies.

In the first place it has been definitely proved that the various growth factors which have appeared to be essential — often in surprisingly small quantities — for the development of certain micro-organisms are nearly all known to act also as accessory food factors in animal metabolism. WILDIERS' bios has been shown to be composed of various factors which could be identified as biotin, thiamine, pyridoxine, inositol, pantothenic acid, nicotinic acid and p.aminobenzoic acid, and for all these compounds it has now definitely been established that they are indispensable food constituents for special types of test animals, on the understanding that their absence in the food either inhibits growth or leads to certain other defects.

A second important viewpoint is that already much light has been thrown on the way in which this mysterious growth factors exert their beneficial action on the cell, apparently many of them, if not all, are essential components of certain enzyme systems. So for instance thiamine has been found to be the active group of the cocarboxylase, the enzyme which decarboxylates pyruvic acid, nicotinic acid is the active group of the dehydrogenase-system, riboflavin is part of one of the respiratory enzymes, and recently it has been proved that a pyridoxine derivative is the active principle in the decarboxylation of amino acids.

An at first sight disturbing factor is that a phylogenetic study of the distribution of the property of auxo-heterotrophy has learnt that on the one hand next to species that are in need of growth factors there are closely related species which can do perfectly without, and that on the other hand the need for a special growth factor is met with in very divergent classes of plants and animals. However, it has been clearly shown that the independence of the so-called auxo-autotrophic organisms of the presence of growth factors in their food is not due to the fact that they can do without these factors, but that they are able to synthesize the compounds in question. This implies that the auxo-heterotrophic cells have obviously lost these synthetic capacities, and such a loss has apparently occurred at very different stages in the course of evolution.

Following SCHOPFER who has made so many important contributions in this field, the situation may be sketched as follows.

One gets the impression that at all levels of evolution one has to do with a living substance that is essentially the same. But during the trend of evolution this living substance has started to lose its independence, the losses in synthetic ability increasing with increasing morphological complexity and increasing adaptation to a heterotrophic life. Yet the main lines of metabolism have been maintained, the ultimate requirements have remained unchanged. The differences between the various physiological groups of organisms are not to be found in differences in the fundamental constitution of their living substance, but just in the different ways in which the latter originates. Whether an organism itself synthesizes the active groups of its enzyme systems, or has to find these groups ready-made in its medium, is only of secondary importance: the final result being the same in both cases.

We may conclude from all this that the study of microbial growth factors in close contact with that of vitamins have also led to an impressive manifestation of a fundamental unity of metabolism in the whole kingdom of life.

On looking back on so many advances in our insight into the principles underlying microbial life one may ask whether future research will not necessarily be restricted to an extension and a consolidation of these findings. It may appear that prospects for further developments of a more essential character are far from bright.

In reality there is already ample evidence that future generations of microbiologists will find vast areas of their science still awaiting cultivation.

Until now I have only been dealing with observations made on micro-organisms as they have been isolated from their natural habitat, and the constancy of their properties during these observations has been a postulate for the discussion of the results obtained. This means that one of the most fundamental characters of every form of life, *viz.*, its variability, has been left out of consideration.

Now it is a common experience of every microbiologist that not rarely a continued study of some micro-organism leads to the conclusion that variations have occurred.

Fortunately in discussing the possibilities inhaerent in microbial variations it is not necessary to have recourse to the still difficultly interpretable results obtained with bacteria. There are other groups of micro-organisms in which because of the occurrence of sexual reproduction the nature of the variation phenomena is open to a detailed analysis.

Amongst the various studies made in this field the series of investigations in recent years carried out by BEADLE and his collaborators at Stanford University on the biochemical genetics of the mould *Neurospora crassa* may be deemed to be outstanding, and there are even scientists who expect that this mould will in the course of time supersede *Drosophila* from its central position in genetics. How this may be, the said investigations are in any case apt to give a clear idea of the importance which induced mutations may well obtain in the future development of microbiology. It is for this reason that it seems worth while to spare some moments for the discussion of some of the results obtained.

Neurospora crassa, commonly known as red bread mould, is heterothallic, that is, exists in two morphologically identical but physiologically different sexes. Each of these is haploid and by itself reproduces only vegetatively by mycelial growth or through the mediation of asexual spores. If mycelia of the two sexes are grown together on a suitable medium hyphal fusions accompanied by nuclear fusions occur and out of the diploid zygotes ultimately asci containing 8 linearly arranged ascospores are formed. Owing to the meiosis occurring in the ascus preceding the ascospore formation the ascospores are again haploid. If the parent strains differ in the alleles of a single gene, the eight ascospores of an ascus are of two kinds, four like one parent and four like the other. It is evident that this situation provides a means to check whether a property characteristic for some induced mutant is dependent on the mutation of a single gene.

BEADLE and his collaborators have now succeeded in obtaining a large number of biochemical mutants by treating asexual spores with X-rays or ultraviolet light, making crosses with strains of the opposite sex, and then establishing genetically homogeneous, single ascospore strains. Special attention has now been given to the question whether these strains differed in their growth requirements from the original, wild-type strain. For the latter it had previously been established that it grew quite satisfactorily in a medium containing some sugar, the necessary inorganic salts — nitrate as a source of nitrogen — and biotin as the only indispensable growth factor. For many of the mutants obtained it was found that they no longer developed in this minimal medium, but that they did so perfectly well if some extract containing the various amino acids and B-vitamins was added. This result can only be understood on the basis of the assumption that the ability of the wild-type strain to synthesize one or more of the essential amino acids and B-vitamins was lost in the mutation act. By composing a series of media, in each of which a single amino acid or one of the growth factors had been added to the minimal medium, and observing in which case growth occurred after inoculation with the mutant strain, it was, of course, possible to establish for this particular strain the specific loss in synthetic ability. By this procedure it could be shown that amongst the numerous mutants obtained there were strains which differed from the wild-type strain by loss of the ability to synthesize one of the following compounds: thiamine, pyridoxine, p-aminobenzoic acid, pantothenic acid, inositol, nicotinic acid, choline, arginine, lysine, leucine, valine, methionine, tryptophan, proline and threonine. Moreover, the genetical analysis showed that each of these strains differed from the wild-type by a single gene, the normal allele of which is evidently essential to the biosynthesis of the compound in question.

A further biochemical investigation of some of these mutant strains has led to some extremely interesting results. It is clear that the biosynthesis of each of the compounds mentioned depends on a long chain of individual step-reactions, and that the blocking of any of these step-reactions will suffice to prevent the synthesis of the compound in question. Since there were already several indications that each particular step-reaction is determined by one gene, it might be expected that mutants failing in the synthesis of one and the same essential compound may differ mutually in the nature of the step-reaction blocked.

This expectation has now been amply confirmed in an investigation of a great number of mutants deficient in their ability to synthesize the amino acid arginine. Amongst these not less than 7 different types have been recognized. Independent biochemical evidence had made it probable that the synthesis of arginine would proceed in this way that first a still unknown series of reactions would lead to the formation of ornithine which then was converted into citrulline which then in a final reaction would yield arginine. It was therefore tested in how far either ornithine or citrulline could meet the growth requirements of the various ,,arginine-deficient'' strains. It was then found that for one mutant these compounds could not be used as a substitute for arginine itself. Evidently here the final step-reaction had been blocked. For a second type of mutants citrulline was acceptable, but ornithine not, indicating a blocking of the ornithine-citrulline step-reaction. For the remaining strains both ornithine and citrulline were suitable substitutes for arginine which must imply that one of the step-reactions in the long chain leading to ornithine synthesis had been blocked. Since theses trains differed mutually, it was obvious that here too in each of the strains different step-reactions were inactivated.

In the first place these results form a strong argument for the one-gene-one-enzyme concept which in slightly different terms was already so passionately advocated by my famous predecessor BEIJERINCK as long ago as 1917. But it is also clear that the production and investigation of biochemical mutants form a powerful tool for the analysis of anabolic processes in the microbial cell. It will be clear that in many cases it will be possible to submit the correctness of assumptions regarding the way in which various cell constituents are synthesized to an experimental test by studying the behaviour of special mutants towards supposed intermediate products.

It is in this way that TATUM, BONNER and BEADLE have made it very probable that the ultimate step in tryptophan synthesis is the condensation of indole and serine, and that moreover anthranilic acid is an immediate precursor of indole.The latter conclusion is based on the fact that if one of the tryptophan-deficient mutants is grown in the presence of a minimal amount of tryptophan the mutant accumulates anthranilic acid and excretes it in the medium from which it has been isolated in the crystalline state.

Here we have the interesting example of a biochemical mutant forming a metabolic product quite unknown for the original type. Is it not tempting to suggest in this connection that the superiority in penicillin production of several mutant strains of *Penicillium notatum* obtained by radiation with X-rays or ultraviolet light, or by bombardment with neutrons, is due to the inactivation of a gene determining an enzyme by which in the wild strains penicillin is converted into some other cell constituent?

However this may be, it is not doubtful that the microbiologist of the future will be able to produce microbes with potencies differing markedly from those known up to this time. And the case of penicillin and other antibiotics is there to testify that the production of even minute quantities of metabolites with new properties may become of far-reaching importance for mankind.

Our conclusion then must be that the coming generations of microbiologists, far from being pitiable, will experience joys both from an increased insight into the mysteries of microbial life and from an increased mastery of its manifestations. And the industrial microbiologist will add one key industry to those already existing: the industry in which microbes with desired qualities will be fabricated!

The lecturer is, of course, aware of the danger inhaerent in such prophecies, and for this reason it seems high time to return to reality. And then I think I may not conclude without devoting a few special words to the progress made regarding our knowledge of that group of micro-organisms which was nearer to EMIL CHRISTIAN

HANSEN's heart than any others: the yeasts. It is in this domain that his name is rightly perpetuated in the generic names *Hansenula* and *Hanseniaspora*.

If at this moment he could have been with us, what would he say? Would he be satisfied with the way in which we his successors have taken care of the heritage he left to us?

After all that I have learnt about the character of this great scientist I do not expect that he would be exuberant in his praise. It is well-known that he feared that such praise might lead to relaxation, instead of acting as a stimulant to further efforts.

But, nevertheless, I venture to think that in his heart a feeling of satisfaction would not be lacking. In the first place this seems to be most probable, when we remind ourselves of the fact that during his life-time HANSEN had to devote part of his energy to the defence of the view that the yeasts which he brought together in the genus *Saccharomyces* have to be considered as independent forms of life, and not, as was repeatedly suggested, are mere stages in the life cycle of higher fungi. Seen in this light HANSEN would have relished in the present situation in which about 100 different ascosporeforming yeast species, divided over 15 genera, are generally accepted as independent organisms in the botanical world. Moreover, he might have rejoiced at seeing that attempts to deal in a somewhat comprehensive way with the systematics of yeasts, of which systematics the foundations were laid by him, have necessitated the publication of three more or less stately volumes.

In view of his great devotion to the industrial applications of yeasts, HANSEN would also have been interested to see that the organisms which are undoubtedly responsible for the particular qualities of special types of English beers continue to receive the attention of scientists of the small nations. Herewith I refer to the remarkable group of yeasts first discovered and described by your fellow-countryman HJELTE CLAUSSEN, and to which the name of *Brettanomyces* was given by him. Later on they have once more been most thoroughly studied in Copenhagen by SCHIÖNNING, and I am glad to be able to add that my collaborator CUSTERS of late could prove that British conservatism has continued to safeguard during another 40 years the hospitality offered by British breweries to a group of micro-organisms, the natural occurrence of which is still wrapped in mystery.

There also seems no doubt that it would be a source of true satisfaction to HANSEN to see that nowadays the industrial application of yeasts is no longer restricted to his two favourite species: *Saccharomyces cerevisiae* and *Saccharomyces carlsbergensis*, but that for the production of food yeast, fat or vitamins several other species have been, or are likely to be introduced into industry in a near future.

But all these minor joys which HANSEN might experience, if he was able to inspect his old kingdom, would appear to him as mere trifles after having given due attention to what had been achieved in his own beloved Carlsberg Laboratory. There he would be confronted with the situation that the president of the EMIL CHRISTIAN HANSEN ,,Fond" had revolutionized his science and opened wide prospects for future developments by applying the mighty tool of genetics to the study of the yeasts. Undoubtedly the fact that it has been possible to produce consciously new strains with interesting properties by hybridisation would have quite especially appealed to the man who already in 1891 published the first observations, at that time not well interpretable, on ascospore copulation in *Saccharomyces cerevisiae*. The idea that the phenomenon of variability in *Saccharomycetes*, to which he already half a century ago had devoted so much attention, has at last successfully been submitted to a careful analysis, and gradually is giving up its secrets, would be another reason for rejoicings.

The realisation that just like in his own days the Carlsberg Laboratory is still the world's leading centre in yeast science would make even HANSEN's stern character break with its principles, and we may presume that he would be unable to withhold words of praise and satisfaction.

(Parasitological Department of the Laboratory of Hygiene, Bacteriology and Tropical Hygiene, University of Leiden).

COMPARATIVE INVESTIGATIONS INTO THE INFLUENCE OF THE PRESENCE OF BACTERIA ON THE LIFE OF PATHOGENIC AND APATHOGENIC LEPTOSPIRAE

by

R. ABDOELRACHMAN

(Received June 6, 1946).

The pathogenic leptospirae which have entered the bodies of rats, apparently find in the kidneys the most favourable conditions for their further existence; according to researches by Ido and his co-workers in 1916 (6) and later on an important percentage of rats appears to be infected with these micro-organisms.

But scarcely have they left the kidneys of their host along with the urine, or the conditions for their further existence become more unfavourable; Noguchi's experiments carried out in 1918 (8) and the experiences gained by later investigators have proved, that acidly reacting urine checks the growth of *Leptospira icterohaemorrhagiae*. The harmful influences increase as soon as they come to live saprophytically on or in soil or in surface water. Apart from physical conditions such as drying and sun-heat (9), the percentage of salt and the pH of the water (10), the presence in their surroundings of microbes: bacteria, water leptospirae and protozoa might also inhibit their growth and development.

According to Appelman's investigations (3) in a combined cultivation of pathogenic and apathogenic saprophytic (water-) leptospiral strains the former die after a short period while the latter keep alive. In 1934 van Thiel (13) has shown that a weak antagonism also exists between virulent and avirulent pathogenic strains, whilst Mochtar's experiments in 1939 (7) show, that after inoculation of a guinea-pig by two different pathogenic leptospiral strains one strain will inhibit the development of the other. These facts, however, need not entail that similar phenomena will occur under natural conditions.

Of greater importance than such antagonisms observed in culture tubes might be the influences on the leptospirae exercised in the open by the numerous bacteria living in soil and surface water. These bacteria are partly autochthonous and for another part have been originally derived from the excreta of men and animals. Not only in free nature but also in contaminated leptospiral cultures in the laboratory leptospirae along with bacteria will occur and will act one upon the other.

Already in 1916, shortly after the discovery of *L. icterohaemorrhagiae* by INADA and his co-workers, NOGUCHI studied experimentally in how far its development would be disturbed by several bacteria introduced in the culture tubes. For these experiments he used culture tubes containing 1.5 ml of rabbit serum, 4.5 ml of Ringer's liquid, 1 ml of citrated plasma and 1 drop of defibrinated rabbit blood, a medium very suitable for leptospirae. The cultures of *L. icterohaemorrhagiae* were inoculated with various bacteria and incubated at 26° C. He used 4 inhabitants of the normal intestine, 5 pathogenic intestinal bacteria, 10 different streptococci, 4 pneumococci of Type I, II, III and IV and 9 types of bacteria not living in the animal body. He ascertained that the more abundantly the bacteria grew, the poorer the leptospirae developed and that the leptospirae were not proof against the harmful influence of bacteria, for whilst in the control tubes without bacteria, the leptospirae were still alive after some weeks, in the other culture tubes they had all died within two weeks. The leptospirae which were brought together with *Bacterium faecalis alcaligenes* remained alive longest, *viz.*, up to 12 days; in most of the tubes with streptococci the leptospirae could still be detected after 3 to 8 days, whilst by the other 3 normal intestinal bacteria and by the pathogenic bacteria they had been destroyed already within 1—3 days. The bacteria not derived from the intestines killed the leptospira already within 24 hours.

In 1921 MANTEUFEL (5) studied the influence of *Bacterium coli*, *Vibrio Metschnikoff* and *Bacterium faecalis alcaligenes* on leptospiral cultures. He stated, that the presence of the vibrios checked the development of *L. icterohaemorrhagiae*, whilst on the other hand the presence of *B. faecalis alcaligenes* had such a favourable influence on the rate of multiplication of the leptospirae, that MANTEUFEL suggests a „symbiosis" between both micro-organisms.

The first investigator, who compared Weil and water leptospirae as to their reaction on the presence of various bacterial species was SHIGA (11). He cultivated either leptospiral type together with *Bacterium faecalis alcaligenes*, *Bacillus megatherium*, *Bacterium violaceum*, *Bacterium prodigiosum* and water vibrios. In the presence of *B. faecalis alcaligenes* both leptospiral species grew well; a particularly abundant growth which might be ascribed to a symbiontic influence of the bacteria could not be stated. He noticed, however, that though *Spirillum volutans* grew poorly in the medium used, its presence had a checking influence on the development of species of leptospirae and that the latter were overgrown by *B. prodigiosum*.

Suchlike experiments were carried out by ANJOW (1) in 1926 in order to compare the resistance of pathogenic and apathogenic leptospiral strain against some types of bacteria. He studied 9 pathogenic and 2 water leptospiral strains as to their behaviour in the presence of 4 strains of *Bacterium coli*, 1 strain of *Streptococcus haemolyticus*, 1 strain of *Bacterium typhosum* and 1 strain of *Bacterium paratyphosum* and arrived at the following results:

The pathogenic and water leptospirae reacted similarly on the presence both of *B. coli*, being killed in half a week and of *Streptococcus haemolyticus*, which did not harm either of them. In the presence of *B. coli communis* and *B. coli communior*, *B. typhosum* and *B. paratyphosum* the leptospiral strains mutually differed in behaviour. Whilst the pathogenic leptospirae died already after 1 or 2 weeks, the water leptospirae were still alive after two weeks so that the pathogenic leptospirae appeared to be less proof against normal intestinal *B. coli* and pathogenic intestinal bacteria than the water leptospirae derived from surface water.

Taylor and Goyle (12) have also noted that pathogenic and saprophytic leptospirae differ in behaviour in the presence of bacteria. They record: „Human strains of leptospirae disappear rapidly from cultures in artificial media when contaminated with other organisms, while water strains in our experience continue to grow well in our contaminated media".

It is well known to investigators of leptospirae that the growth may be checked by bacterial contamination of cultures of pathogenic leptospirae. Uhlenhuth and Fromme (14), Zuelzer (16), Schüffner (10), Kathe (4) and others affirm this fact. With regard to the influence of bacteria on the growth of water leptospirae such agreement is lacking however. Whilst most of the investigators are of the opinion that the water leptospirae are more proof against it than the pathogenic ones, Zuelzer maintains that both groups do not differ in the least in this respect (15). Both leptospiral types will grow together in the same culture medium, both types behaving outside the animal body quite similarly, so that the presence of water leptospirae should be considered as an indicator for the occurrence of Weil leptospirae. So it was deemed desirable to investigate more closely the influence of bacteria on leptospirae.

Experimental part.

From January up to April 1942, from August up to November 1942 and from October 1943 up to March 1944 investigations were carried out by van Thiel, Nubé and the author, with the aim of studying the effect of bacteria occurring along with leptospirae in free nature. Difficulties caused by war conditions brought these experiments untimely to a close. It was meant to study the symbiosis of the micro-organisms not only in culture tubes, but also by means of combined cultivation so as to approach natural conditions as much as possible. Only the former part of the investigation could be finished, the latter part will be carried out as soon as circumstances will permit.

The development of pathogenic and water leptospirae (*L. biflexa*) was compared in the presence of bacteria which will occur frequently in their natural surroundings, *viz.*, bacteria derived from water, from soil, from the normal human stool and pathogenic intestinal bacteria of the typhi- and paratyphi group. The Korthof medium [1]) has been used which is particularly suitable for these organisms and in which the various bacteria thrive as well.

In order to keep evaporation down the cotton plugs have been paraffined. The cultures were transferred weekly.

In the first experimental series three pathogenic strains of Leptospira were studied as to their behaviour in the presence of *Mycobacterium rubrum,* a soil organism. The results are recorded in Table I.

Table I.

Pathogenic leptospirae cultivated together with *Mycobacterium rubrum*

Temperature of incubation	Duration of life in weeks		
	L. icterohaemorrhagiae strain Coppée	*L. icterohaemorrhagiae* strain Muyzert	*L. canicola* strain Polly
room temperature	2—3	8	—
28—30° C.	6	>14	6

In the second experimental set the behaviour of three pathogenic and three apathogenic strains of Leptospira in the presence of various bacteria was studied. Both apathogenic and pathogenic bacteria were introduced into the cultures. The results are taken up in Table II.

These preliminary experiments carried out by van Thiel and Nubé allow of the following conclusions:

1°. Water leptospirae grow at room temperature as well as at 28°—30° C. and are not disturbed in their development by the bacteria introduced into the culture.

2°. Pathogenic leptospirae develop well at 28—30° C. and are then not injured by the presence of bacteria. When cultivated, however, at room temperature together with bacteria, they die off within 1—5 weeks. Also when cultivated without the presence of bacteria, room temperature is less suitable for pathogenic leptospirae. Thus in the third experimental set all cultures have been incubated at 28—30° C.

---

[1]) Peptone (Witte) 400 mg, sodium chloride 700 mg, sodium bicarbonate 10 mg, potassium chloride 20 mg, calcium chloride 20 mg, potassium phosphate dibasic cryst. 90 mg, sodium phosphate 485 mg, dest. water 500 cc + 10 per cent rabbit serum.

Table II.

Leptospiral strains cultivated together with various bacteria

Temperature of incubation	Duration of life in weeks			
	Pathogenic			Apathogenic
	L. icterohaemorrhagiae strain Coppée	*L. icterohaemorrhagiae* strain Muyzert	*L. canicola* strain Polly	*L. biflexa* strain I, IX, X
	With *Mycobacterium rubrum*			
room temp.	3—5	1—3	—	>5
28—30° C.	>5	>5	—	>5
	With *Bacterium coli* 46			
room temp.	1—3	1	—	>5
28—30° C.	>5	>5	—	>5
	With *Bacterium coli* 280			
room temp.	1—3	—	—	>5
28—30° C.	>5	—	—	>5
	With *Bacterium typhosum*			
room temp.	1	1—2	1	>5
28—30° C.	>5	>5	>5	>5
	With *Bacterium paratyphosum* A			
room temp.	2—4	—	1	>5
28—30° C.	>5	—	>5	>5
	With *Bacterium paratyphosum* B			
room temp.	1	—	1	>5
28—30° C.	>5	—	>5	>5

Third experimental set. The agglutination tubes filled up till half with Korthof medium were supplied with 1 drop of a culture of *L. biflexa* or 2 drops of the pathogenic leptospiral cultures, this because of the twice as abundant growth of the former. The culture tubes were provided with 1 drop of the various bacterial suspensions in physiological salt solution. The growth of the leptospirae and of the bacteria was examined weekly in dark field illumination. Next to the amount of leptospirae their degree of motility was noted. The examination of the cultures was continued until either the leptospirae had disappeared completely, or in case they kept alive up till the 6th—12th week.

3 pathogenic leptospiral strains were examined, *viz.*, *L. icterohaemorrhagiae* strain Wijnberg, *L. grippotyphosa* strain Andaman,

L. canicola strain Polly and 3 water strains, ***viz.***, *L. biflexa* strain I, isolated from tap-water in Leiden and the strains VIII and X isolated from ditch water near Leiden (van Thiel, 1933). The strains Coppée, Destreberg and Muyzert used in the earlier experiments had got lost.

The bacteria [1]) used in this experimental series are: As a soil organism, *Mycobacterium rubrum*. As water bacteria, *Pseudomonas putida*, *Ps. fluorescens*, *Ps. punctata*, *Spirillum serpens*, *Micrococcus albus* and *Azotobacter agilis*. As normal intestinal bacteria, *B. coli* 46 and 280, *Aerobacter cloacae* and *Streptococcus faecalis*. As pathogenic intestinal bacteria, *B. typhosum*, *B. paratyphosum* A and B.

In Table III the results of these experiments are recorded.

Discussion.

Soil bacteria. In the presence of *Mycobacterium rubrum* the pathogenic as well as the water leptospirae grow abundantly for some weeks; 4 to 10 weeks after the inoculation they had all died off and no difference could be noted between both leptospirae groups.

Water bacteria. In the presence of the 3 species of *Pseudomonas* and of *Sp. serpens*, all pathogenic leptospirae died off in one week. *Micrococcus albus* varied in action on the three leptospiral strains, whilst *Azotobacter agilis* acted very mildly on all of them.

As to the apathogenic leptospirae, the 3 species of *Pseudomonas* and *Sp. serpens* are very harmful for *L. biflexa* str. I and X, whilst str. VIII stands *Ps. putida* much better. *Micrococcus albus* and *Azotobacter agilis* are less harmful although their action on the various strains of *L. biflexa* varies.

So *Azotobacter agilis* does not or merely after a long period act harmfully on the growth both of pathogenic and apathogenic leptospirae. In the presence of the other water bacteria the apathogenic leptospirae generally appear to remain alive longer than the pathogenic species.

Normal intestinal bacteria. *Aerobacter cloacae* is very injurious to the growth of pathogenic leptospirae. *Streptococcus faecalis* either does not act or very mildly and the *B. coli* strains vary markedly in action on this leptospirae group.

As to their influence on apathogenic leptospirae, *B. coli* and *Aerobacter cloacae* vary greatly in their action. In the presence of *Streptococcus faecalis*, however, the leptospirae can still be detected after 9 weeks.

So with regard to the normal intestinal bacteria no clear-cut differences exist between the pathogenic and apathogenic leptospirae.

---

[1]) Professor Kluyver in Delft was ready to place many of the here mentioned bacterial strains at our disposal.

Table III.

Occurrence of leptospirae in the microscopical field (temperature of incubation 28—30° C.).

Weeks passed [1]		1	2	3	4	5	6	7	8	9	10
Organism introduced in culture						*Mycobacterium rubrum*					
L. icterohaemorrhagiae	+	++++	+++	+++	++	+	—[3]				
L. grippotyphosa	+	++	+++	+++	+	—[2]					
L. canicola	+	+++	+++	+++	+++	+	—[2]				
L. biflexa str. I	+	++++	++	+	—[2]						
L. biflexa str. VIII .	+	++++	++++	+++	++	++	+	—[3]			
L. biflexa str. X	+	+++	++++	++++	++++	+	+	+	+	+	—[3]
						Pseudomonas putida					
L. icterohaemorrhagiae	+	—									
L. grippotyphosa	+	—									
L. canicola	+	—									
L. biflexa str. I	+	+	—								
L. biflexa str. VIII .	+	++	++	++	+	+	+	+	+[3]		
L. biflexa str. X	+	—									
						Pseudomonas fluorescens					
L. icterohaemorrhagiae	+	—									
L. grippotyphosa	+	—									
L. canicola	+	—									
L. biflexa str. I	+	—									
L. biflexa str. VIII .	+	++	+	+	—[2]						
L. biflexa str. X ...	+	—									

Table III (continued)

Weeks passed 1)		1	2	3	4	5	6	7	8	9	10
Organism introduced in culture	*Pseudomonas punctata*										
L. icterohaemorrhagiae	+	—									
L. grippotyphosa	+	—									
L. canicola	+	—									
L. biflexa str. I	+	—									
L. biflexa str. VIII .	+	+	—								
L. biflexa str. X	+	—									
	Spirillum serpens										
L. icterohaemorrhagiae	+	—									
L. grippotyphosa. . . .	+	—									
L. canicola	+	—									
L. biflexa str. I	+	—									
L. biflexa str. VIII .	+	+	+	— 3)							
L. biflexa str. X	+	—									
	Micrococcus albus										
L. icterohaemorrhagiae	+	—									
L. grippotyphosa	+	+ +	+ + +	+ +	+	+	—				
L. canicola	+	+ + +	+ +	+	—						
L. biflexa str. I . . .	+	+	—								
L. biflexa str. VIII .	+	+ + +	+ + + +	+ +	+ +	+	+	+	+ 3)		
L. biflexa str. X	+	+ +	+ + +	+	—						

Table III (continued)

Weeks passed 1)		1	2	3	4	5	6	7	8	9	10
Organism introduced in culture											
					Azotobacter agilis						
L. icterohaemorrhagiae	+	++++	++++	++++	++	+	+				
L. grippotyphosa	+	+++	+++	++	+	—					
L. canicola	+	+++	+++	+++	+++	+++	+	— 2)			
L. biflexa str. I	+	++++	+++	+	—[3]						
L. biflexa str. VIII .	+	+++	++++	+++	++	+	—				
L. biflexa str. X	+	++++	++++	+++	+++	+++	++	+	+	— 2)	
					Bacterium coli 46						
L. icterohaemorrhagiae	+	+	+	—							
L. grippotyphosa	+	+	—								
L. canicola	+	+	+	+	+	—					
L. biflexa str. I	+	+	—								
L. biflexa str. VIII .	+	+	+++	++	+	+	+	+	+[3]		
L. biflexa str. X	+	++	+++	++	+	+	+	+	—[2]		
					Bacterium coli 280						
L. icterohaemorrhagiae	+	+	+	+	—						
L. grippotyphosa	+	++	+++	+++	++	+	+	+	— 2)		
L. canicola	+	+	+	+	+	+	+	—			
L. biflexa str. I	+	+	+	—							
L. biflexa str. VIII .	+	—									
L. biflexa str. X	+	+	+++	++	+	+	+	—			

Table III (continued)

Weeks passed [1]		1	2	3	4	5	6	7	8	9	10
Organism introduced in culture	*Aerobacter cloacae*										
L. icterohaemorrhagiae	+	—									
L. grippotyphosa	+	—									
L. canicola	+	—									
L. biflexa str. I	+	—									
L. biflexa str. VIII .	+	+	+++	+	+	+	+	+	+ [2]		
L. biflexa str. X	+	—									
	Streptococcus faecalis										
L. icterohaemorrhagiae	+	++++	++++	++++	+++	+++	+++				
L. grippotyphosa	+	+++	+++	+++	+++	++	+	+	+ [2]		
L. canicola	+	++	+++	+++	++	+	+	+	+ [2]		
L. biflexa str. I	+	++++	++++	++++	+++	+++	+++				
L. biflexa str. VIII .	+	++++	++++	+++	+++	++	+	+	+ [3]		
L. biflexa str. X	+	++	++	++	+++	+++	++	++	++	+ [3]	
	Bacterium typhosum										
L. icterohaemorrhagiae	+	—									
L. grippotyphosa	+	+	—								
L. canicola	+	—									
L. biflexa str. I	+	—									
L. biflexa str. VIII .	+	++	++	++	+	+	+	+	+	— [2]	
L. biflexa str. X	+	+	+++	++	++	+	+	+	+	+	+ [2]

Table III (continued)

Weeks passed[1]		1	2	3	4	5	6	7	8	9	10
Organism introduced in culture	*Bacterium paratyphosum* A										
L. icterohaemorrhagiae	+	—									
L. grippotyphosa	+	—									
L. canicola	+	+	—								
L. biflexa str. I	+	+ +	—								
L. biflexa str. VIII .	+	+	+ +	+	+	+	+	—[2]			
L. biflexa str. X	+	+ +	+ +	+ +	+ +	+ +	+ +	+	+	+	+
	Bacterium paratyphosum B										
L. icterohaemorrhagiae	+	—									
L. grippotyphosa	+	—									
L. canicola	+	—									
L. biflexa str. I	+	+ +	—								
L. biflexa str. VIII .	+	+ + +	+ + +	+ +	+	+	+	—[2]			
L. biflexa str. X	+	+	+	+	+	+	+	+	+	+	+[2]

— = no leptospirae.
+ = some leptospirae per microscopical field.
+ + = more ,, ,, ,, ,,
+ + + = many ,, ,, ,, ,,
+ + + + = the microscopical field is full of leptospirae.

---

[1] In this table only the series of experiments is mentioned, where the culture tubes are not transferred. The cotton plugs are coated with paraffin.

[2] When weekly transferred some (+) or a few (+ +) leptospirae are found per microscopical field.

[3] When weekly transferred many (+ + +) leptospirae are found per microscopical field.

Pathogenic intestinal bacteria. The bacteria tested are equally harmful for the pathogenic leptospirae. Among the apathogenic *L. biflexa,* strain I is very sensitive to their presence, whilst str. VIII and str. X appear to be proof to it.

So among the 6 leptospirae strains studied, *L. biflexa* str. VIII is most proof against the influence of the various bacterial types; *L. biflexa* str. X is less proof than str. VIII. Of the apathogenic leptospirae strains *L. biflexa* str. I behaves like the pathogenic types. *L. icterohaemorrhagiae* strain Wijnberg of all strains tested is mostly injured by the presence of bacteria. *L. grippotyphosa* strain Andaman and *L. canicola* strain Polly are also sensitive although in a lesser degree than the former.

Thus it may be concluded that Zuelzer is wrong in her opinion that no difference would exist between pathogenic and apathogenic leptospirae as far as their behaviour towards bacteria is in question. It is true that sometimes they may behave equally, but in other cases they differ. Moreover differences appear to exist between different strains of one type of leptospira.

As to the bacterial influence in free nature the possibility exists that:

a. the soil bacterium *Mycobacterium rubrum* may be injurious for leptospirae after a lapse of time.
b. the water bacteria *Ps. putida, Ps. fluorescens* and *Ps. punctata* and *Sp. serpens* will soon destroy the leptospirae, more especially the pathogenic species.
c. the normal intestinal bacteria check the growth of leptospirae.
d. the pathogenic intestinal bacteria of the typhi paratyphi-group are more injurious for the pathogenic leptospirae than for the apathogenic leptospirae types.

Bibliography.

1. S. Anjow, Z.f. Imm. Forschung **109**, 61, 1928. – 2. J. M. Appelman und P. H. van Thiel. Zentralbl. f. Bakt.I, **133**, 224, 1934. – 3. J. M. Appelman, Het isoleeren van *Leptospira icterohaemorrhagiae*, Diss. Leiden, 1934. – 4. J. Kathe, Erg d Hyg Bakt. Imm. u. exper. Ther. **24**, 159, 1941. – 5. P. Manteufel, D. Med. Wschr. **47**, 461, 1921. – 6. Y. Ido and co-workers, J. of exp. Med. **24**, 471, 1916. – 7. A. Mochtar, Z. f. Imm. Forsch. **95**, 523, 1939. – 8. H. Noguchi, J. of exp. Med. **27**, 609, 1918. – 9. Th. O. E. Polanen, Diss. Utrecht, 1939. – 10. W. Schüffner, Transact. R. Soc. Trop. Med. **28**, 7, 1934—1935. – 11. A. Shiga, Z.f. Imm. Forsch. **40**, 148, 1924. – 12. J. Taylor and A. N. Goyle, Ind. Med. Res. Mem. **20**, 000, 1931. – 13. P. H. van Thiel, Antonie van Leeuwenhoek **4**, 237, 1934. – 14. P. Uhlenhuth und W. Fromme, in W. Kolle und A. Wassermann, Handb. d. path. Mikroorganismen. 1930. – 15. M. Zuelzer, Zentralbl. f. Bakt I, **136** 194, 1930. – 16. M. Zuelzer, Handb. d. path. Prot. von S. von Prowazek, 1931.

(From the Microbiological Laboratory of the Zuiderzee Reclamation Works,
Kampen, Holland).

THE INFLUENCE OF THE OXIDATION-REDUCTION POTENTIAL OF THE MEDIUM UPON THE GROWTH OF *AZOTOBACTER CHROOCOCCUM*

by

A. QUISPEL

(Received August 22, 1946).

1. INTRODUCTION.

Though all investigators agree that the harmful effect of oxygen on anaerobic bacteria is mainly caused by the high redox potential produced (by the oxygen), opinions are divided on the question whether the beneficial effect of oxygen on aerobic organisms is attributable to the same cause. While the experiments of BALDWIN c.s. (2, 3, 4, 5) show clearly that the development of aerobic organisms is influenced by the redox potential of the medium, KNAYSI and DUTKY (9) gave it as their opinion that for aerobic bacteria oxygen as such cannot be substituted by other redox systems, and that the harmful effect of low redox potentials is produced by the corresponding low oxygen tension. The relationship of aerobic bacteria to the redox potential of the medium is of interest not only in itself, but also as a possible method of testing the suitability of the redox potential of the soil for the growth of higher plants. Therefore we decided to investigate this problem. The experiments were only of a preliminary nature, but in spite of this some important conclusions could be drawn.

Our experiments were performed with one of the most aerobic bacteria, *Azotobacter chroococcum*, which in nature appeared to be very dependent on the aeration of certain soils. The bacterium was isolated from a garden soil in Kampen. As a culture medium we always used the following standard solution of BORTELS: mannitol 2 %, K_2HPO_4 0.1 %, $CaCO_3$ 0.1 %, $MgSO_4 \cdot 7H_2O$ 0.05 %, $FeSO_4$ 0.005 %, $MnSO_4$ 0.001 %, Na_2MoO_4 0.001 %. For the preparation of agar media 2 % agar was added to this solution.

2. EXPERIMENTS WITH AGAR CULTURES.

According to ALLYN and BALDWIN (2) aerobic bacteria show their main development in agar shake cultures at a definite level below the surface of the agar. This level can be raised or lowered by the addition of reducing or oxidising substances, showing the dependence of the bacteria on the oxidation-reduction character of the medium. As the level of optimal development could be markedly lowered by the addition of oxidising substances, these workers even suggested the possibility that aerobic bacteria might develop under anaerobic conditions with the addition of oxidising substances.

We tried to use this method in studying the effect of the redox potential of the medium upon the growth of *Azotobacter chroococcum*. The following culture media were used:

1: the normal standard solution with 2 % agar.

2: The same solution in which the mannitol had been replaced by glucose.

3: the normal standard solution without Ca.

4: the glucose standard solution without Ca.

Of course the solution with glucose was more reducing than the solution with mannitol. In some experiments the medium was made still more reducing by the addition of hydroquinone or ascorbic acid.

As oxidising substances $KMnO_4$, $KClO_3$ and H_2O_2 were used. Of these, only $KMnO_4$ gave reliable results. The two other oxidising substances were toxic even in very low concentrations.

The results of ALLYN and BALDWIN could not be reproduced. No development was observed either in media which were too reduced or in those which were too oxidised. In media with a good oxidation-reduction character, a more or less abundant development of *Azotobacter* was observed at the surface of the agar. When the medium was too reduced or too oxidised to allow full development of the pellicle at the surface, but not sufficiently so as to suppress all growth a small number of colonies developed in the agar. These subsurface colonies were only found when no pellicle was formed *i.e.*, the development of colonies beneath the surface of the agar was not dependent on the redox potential of the medium but only on the absorption of gases from the atmosphere by bacteria at the surface. As the same effect could be observed in media containing NH_4NO_3, we must assume that *Azotobacter* cannot develop in the absence of oxygen.

However, the pellicle at the surface appeared to be highly dependent on the redox character of the medium. Some of the most important results are summarized in table I.

From this table it is evident that the development of *Azotobacter* is markedly influenced by the reducing conditions of the medium. The more reducing the character of the medium, the higher must be the concentration of $KMnO_4$ to permit an optimal development of *Azotobacter*. High concentrations of $KMnO_4$ are inhibitory, due either to establishment of too high potentials or to chemical toxicity. The presence of Ca enables the bacteria to withstand a greater range of potentials.

3. EXPERIMENTS IN LIQUID CULTURE SOLUTIONS.

As in the preceding experiments by the addition of oxidising substances no growth in the agar could be obtained there was

Table I.

The influence of the oxidation-reduction character of the medium upon the development of *Azotobacter* in agar shake cultures.

The numbers in the right part of the table indicate the degree of development of *Azotobacter*: 0 no development; 1 some colonies scattered through the agar; 2 more colonies scattered through the agar, one or two big colonies at the surface; 3 a ring of growth at the surface; 4 a pellicle at the surface, no colonies in the agar; 5 a thick pellicle at the surface. The estimations were performed after incubation for 3 days at 30° C.

source of carbon	percentage of $CaCO_3$	percentage of hydroquinone	percentage of ascorbic acid	no $KMnO_4$	0.001 % $KMnO_4$	0.002 % $KMnO_4$	0.005 % $KMnO_4$	0.01 % $KMnO_4$	0.02 % $KMnO_4$	0.04 % $KMnO_4$	0.08 % $KMnO_4$	0.16 % $KMnO_4$	0.32 % $KMnO_4$
glucose	0.0	0.0	0.0	0	0	1	2	3	4	5	0	0	0
glucose	0.1	0.0	0.0	1	3	3	5	5	5	5	4	0	0
mannitol	0.0	0.0	0.0	3	3	4	5	5	4	2	0	0	0
mannitol	0.1	0.0	0.0	5	5	5	5	5	2	1	0	0	0
mannitol	0.1	0.0001	0.0	1	4	—	5	—	—	0	—	—	—
mannitol	0.1	0.001	0.0	0	0	—	1	—	—	0	—	—	—
glucose	0.1	0.0001	0.0	0	0	—	4	—	—	—	—	—	—
mannitol	0.1	0.0	0.001	0	—	—	4	—	—	—	—	—	—

no further need to perform experiments in agar cultures as the influence of reducing substances could be studied more easily in liquid culture solutions.

For these experiments we used Erlenmeyer flasks of 100 cc capacity filled with 20 cc of the standard solution. The reducing agents were added to this solution after sterilisation and the flasks were inoculated. The preparation of the inoculum required some precautions as otherwise reproducible results could not be obtained. For this purpose, *Azotobacter* was cultivated in Erlenmeyer flasks containing the standard solution. After repeated subculturing a 24 hours culture was shaken to disperse the cells evenly through the liquid, after which it was poured into a test-tube. The suspension was allowed to settle for half an hour, after which some drops of the liquid were inoculated into an Erlenmeyer flask with sterile standard solution. Here the number of cells was counted with a haemocytometer and the suspension diluted with sterile standard solution till one drop of the suspension contained ± 500 cells. The Erlenmeyer flasks of the experimental series then were inoculated each with one drop. The cultures were incubated for three days at 30° C., after which period the development of *Azotobacter* was estimated visually. For certain purposes we thought it desirable to make a more accurate determination of the development of the bacteria; in these experiments the number of cells was measured nephelometrically according to BORTELS. To every culture 5 cc of concentrated hydrochloric acid and 10 cc water was added, after which the cultures were boiled in order to obtain an even distribution of the bacterial cells through the liquid. The cultures were then compared with a control in the normal standard solution which had been inoculated in the same way and then the turbidity was expressed as a percentage of that of the control.

Our first task was to examine the influence of different reducing agents on the development of *Azotobacter* under aerobic conditions, and to compare the inhibitory action of the reducing agents with the redox potential established in the medium.

The redox potential of the culture solutions was measured with electrodes of platinized platinum. We preferred the use of platinized platinum, as it appeared that in poorly poised solutions in which oxygen is the main factor determining the potential, platinized electrodes give more reliable results than electrodes of bright platinum. There remains a possibility that the potentials measured are too low since even platinum does not behave as an indifferent electrode towards oxygen. In well poised solutions *e.g.* in the solutions containing ascorbic acid, hydroquinone etc., both platinized and bright electrodes give the same results. The potentials at the electrodes were measured with a Coleman vacuum-tube potentiometer. The pH was measured with a glass electrode and the same potentiometer. Both Eh and pH were measured in controls at time of inoculation (half an hour after the addition of the reducing substances). Later on a gradual increase of potential in the sterile solutions is observed, as the reducing substances are oxidised by the air.

From the data in table II it is evident that all reducing substances used inhibit the growth of *Azotobacter*. Though it is difficult to see a very close correlation between the inhibiting action and the redox potential measured in the culture solutions, the lowest potential enabling the development of *Azotobacter* is for the most part in the neighbourhood of + 380 — 400 mV.

Table II.

Development of *Azotobacter chroococcum* in media containing reducing substances under aerobic conditions.

++ very good development
+ development slightly retarded
— no development
Eh in mV. The Eh of the control is the mean value of several determinations.

reducing substance	%	Eh	pH	development of *Azotobacter*
control		+ 500	7.52	+ +
hydroquinone	0.0001	+ 471	7.42	+
hydroquinone	0.001	+ 398	7.53	—
ascorbic acid	0.001	+ 459	7.42	+
ascorbic acid	0.01	+ 278	7.28	—
cysteine	0.001	+ 480	7.62	+ +
cysteine	0.01	+ 368	7.30	—
Na_2S	0.001	+ 461	7.60	+
Na_2S	0.01	+ 91	7.81	—
Na_2SO_3	0.02	+ 389	7.73	+
Na_2SO_3	0.04	+ 350	7.89	—
Na_2SO_3	0.1	+ 255	7.89	—

When considering the results of experiments like this we have to remember that reducing conditions only exist during the early part of the incubation period as later on the added reducing substances will have been oxidised by the air. Consequently we might expect development to be merely retarded by the reducing conditions, growth proceeding as soon as the added reducing substances become oxidised. However, this was not the case. For the most part no development at all was observed in the reducing media indicating that the bacteria are killed by the reducing substances.

4. EXPERIMENTS UNDER ANAEROBIC CONDITIONS.

Though *Azotobacter* was suppressed by relatively small amounts of reducing substances under aerobic conditions, it is uncertain whether this is due to the action of the reducing substances upon the redox potential of the medium or to the absorption of oxygen by the reducing substances. As KNAYSI and DUTKI (9) have shown that even under fully aerobic conditions the percentage of oxygen in media with reducing substances may be nil, the toxic effect of the reducing substances might have been due to the absence of oxygen during the prevalence of reducing conditions rather than to an intrinsic toxicity of the reducing conditions themselves.

During the experiments with agar shake cultures we obtained strong evidence that other redox systems cannot replace oxygen for the development of *Azotobacter*. Furthermore, we were unable to cultivate *Azotobacter* under anaerobic conditions in Erlenmeyer flasks containing the standard culture solution with some oxidising substances, thus confirming the results of KNAYSI and DUTKY with *B. megatherium.*

The anaerobic conditions were obtained by incubating the Erlenmeyer flasks with the culture solutions in a desiccator over an alkaline solution of pyrogallol.

As the cells had been killed under aerobic conditions by reducing substances it was interesting to know whether anaerobic conditions too were lethal. To test this possibility, the cultures were taken out of the desiccators after an anaerobic incubation of two days. In flasks of the standard solution with mannitol as a source of carbon, a rapid development of *Azotobacter* was observed as soon as aerobic conditions had been restored. However, when mannitol had been replaced by glucose (which only slightly retards the growth of *Azotobacter* under aerobic conditions), the bacteria appeared to be killed by an anaerobic incubation of only one day.

As it was difficult to measure the redox potential of the media potentiometrically in the desiccators, we added redox indicators to some uninoculated flasks with culture solution. In the solutions with mannitol, dichlorophenole indophenole was not discoloured under anaerobic conditions, showing the potential to be higher than + 230 mV. In the solutions containing glucose, even neutral red was discoloured showing the potential to be lower than —340 mV. Furthermore, the potential of the solution with mannitol was measured potentiometrically in a small beaker closed with a rubber stopper through which an electrode and an agar bridge were inserted. A smaller beaker with the mannitol culture solution was placed in this beaker and the oxygen absorbed by alkaline pyrogallol. It appeared that the potential of the culture solution only decreased by ± 60 mV (Eh = + 440 mV).

From these experiments it is evident that the mere absence of oxygen inhibits the development of *Azotobacter* but does not kill the cells. However, when anaerobic conditions coincide with a low redox potential of the medium, the bacteria are killed.

5. THE INLUENCE OF Ca AND Na ON THE DEPENDENCE OF THE REDOX POTENTIAL.

From the experiments with agar shake cultures, it was obvious that the range of potentials which can be tolerated by *Azotobacter* is considerably increased by the presence of Ca in the medium. The same effect had been mentioned by ALLYN and BALDWIN for *Rhizobium*, while some of the observations of KINGMA BOLTJES (8) might indicate that the same is true of nitrifying bacteria.

We have obtained clear evidence that the effect of ions may be very complex. One of the most interesting experiments is summarized in table III.

Table III.

The development of *Azotobacter* in culture solutions containing ascorbic acid and different concentrations of $CaCl_2$ and NaCl.

The Eh produced by ascorbic acid in this experiment was ± + 300 mV. The development of the bacteria is measured nephelometrically and expressed as percent of turbidity of standard control.

concentration ascorbic acid in %	concentration NaCl in %	concentration $CaCl_2$ in %	pH	development
0	0	0	7.00	100
0.01	0	0	6.65	0
0.01	0.15	0	6.77	0
0.01	0.10	0	6.56	0
0.01	0.05	0	6.96	0
0.01	0.01	0	6.50	0
0.01	0	0.15	5.81	120
0.01	0	0.10	5.63	106
0.01	0	0.05	5.93	90
0.01	0	0.01	6.82	96
0.01	0.10	0.10	6.04	100
0.01	0.01	0.01	6.80	0
0.01	0.10	0.01	6.77	0
0.01	0.01	0.10	5.69	100

The effect of added Ca in this experiment is interesting, since the standard medium (without Ca addition) already contained 0.1 %

$CaCO_3$. The fact that $CaCO_3$ is not effective in this connection shows that only calcium ions in solution can effect the resistence to ascorbic acid.

The fact that microbial requirements for Ca as a nutrient are extremely low, combined with the antagonistic effects of Na ions as shown in table III, indicate that in all probability the added Ca acts here on the colloidal systems of the cytoplasm, either by changeing the permeability of the cell membrane for ascorbic acid or by changeing the specific adsorption of this redox system.

The effect of the ions seems to be still more complex than would appear from the data shown in tabel III. For instance, the addition of NaOH allowed the development of *Azotobacter* in media containing reducing substances within certain limits. For example in media containing 0.1 % ascorbic acid or 0.1 % cysteine (used as hydrochloride) the addition of NaOH, necessary for their neutralisation, allowed the development of *Azotobacter*. The same effect was sometimes observed in media containing 0.1 % alkaline Na_2S. Thus the paradox appeared that substances which were toxic at concentrations of 0.01 % could be endured at concentrations of 0.1 %! The effect of the ionic relations in the medium was often very inconvenient, as impurities of the chemicals used or changes in the water used for the preparation of the culture solutions often greatly influenced the results of the experiments.

We are still far from having a detailed insight into this very complex problem. We can only say that the influence of reducing substances upon the development of *Azotobacter* is highly affected by certain ionic relations in the medium.

6. THE INFLUENCE OF ORGANIC SUBSTANCES ON THE DEPENDENCE OF *Azotobacter* ON THE REDOX POTENTIAL OF THE MEDIUM.

It is well known that the inhibiting action of media which are too highly reduced or oxidised to allow of a normal development of certain bacteria, may be overcome by using a heavy inoculum. This is generally interpreted as being due to the ability of the bacteria to poise the potential in their immediate vicinity. Such an explanation appears reasonable in the case of highly oxidised media. However, as nearly all bacteria markedly decrease the potential of the surrounding medium, it is difficult to understand how a heavy inoculum could be beneficial when the potential of the medium is too reduced.

We observed that in certain circumstances the harmful effect of reducing substances could be overcome not only by using a heavy inoculum but also by the addition of a sterilized filtered culture of *Azotobacter*. This filtrate was prepared from a well developed culture of *Azotobacter* in the normal standard solution and sterilized at 120° C., after which it was added to the culture solution in a concentration of 2.5 % by volume. The effect of this addition is shown in table IV.

Table IV.

The development of *Azotobacter* in the presence of reducing substances and *Azotobacter* filtrate. The development of the bacteria is expressed nephelometrically as percent of turbidity of standard control.

reducing substance	%	*Azotobacter* filtrate in % by volume	pH	Eh in mV	development
control			7.50	520	100
ascorbic acid	0.01	0	7.67	314	0
ascorbic acid	0.01	2.5	7.58	300	142
ascorbic acid	0.01	0.25	7.68	325	136
ascorbic acid	0.01	0.025	7.72	307	0
cysteine	0.01	0	7.30	368	10
cysteine	0.01	2.5	7.42	281	100

The data of table IV show the influence of *Azotobacter* extract to be very pronounced. As its activity was destroyed by ashing, the active materials are organic in nature. We still cannot say whether the filtrate is active by the presence of certain nutritive substances or by the activity of vitamin-like substances. We merely want to stress the fact that growing cells of *Azotobacter* secrete certain substances into the surrounding medium which permit development at lower potentials than would be possible in their absence. This explains how cells of *Azotobacter* help one another to grow at low potentials. This is important, since *Azotobacter* itself markedly decreases the potential in its environment *e.g.* after an incubation period of four days potentials as low as 75 mV could be observed in inoculated culture media kept under aerobic conditions. The potential rapidly increases when metabolic activity is suppressed. The potentials observed in growing cultures of *Azotobacter* were much lower than the potentials which proved toxic in the experiments shown in table II. Though it is possible that the tolerated oxidation-reduction range is widened as the cells grow older (as has been suggested for anaerobic bacteria by VENNESLAND and HANKE (12)) the influence of the substances secreted into the medium will play a role by allowing the cells in the logarithmic phase to live at potentials which would have been toxic during the lag phase. Furthermore, the excretion of these substances might explain the fact that in media with too low a redox potential, heavy inocula may initiate development while light inocula fail to do so.

Discussion.

If the medium and the cell were two systems in thermodynamic equilibrium being in contact with each other through a fully permeable membrane one could expect a close relationship between the redox potential of the medium (which then would be equal to the redox potential of the cell) and the development of the cell. This, however, is not the case. In a medium under anaerobic conditions a thermodynamic equilibrium may be reached, though it may take considerable time for this equilibrium to become established. Under aerobic conditions equilibrium is only reached when all reducing substances have been oxidised by the air. As long as an equilibrium has not been reached, the potential measured at the electrode is mainly determined by kinetic factors and may consequently be highly influenced by catalytic agents. PLOTZ and GELOSO (11) already showed that the potential of anaerobic solutions of glucose can be lowered by the addition of platinized platinum. The same results may be attained by the catalytic activity of the living cell! This is one of the reasons why redox systems may establish a potential in the cell quite different from that in the medium.

There are some other reasons for this belief. ABRAMSON and TAYLOR (1) and KORR (10) have shown that the reduced and oxidised forms of many reversible redox systems are unequally adsorbed by many adsorbing substances resulting in a change in the redox potential. So we may expect that even when a reversible redox system is in perfect thermodynamical equilibrium in the medium the reduced and oxidised forms of this redox system will be unequally adsorbed by the cell colloids, so that within the cell an entirely different potential is established.

It is common knowledge that the living cell as a whole is not in thermodynamic equilibrium. The determination of the redox potential of the cell as performed by many authors is useless, since the redox potentials in the cell differ from point to point. The cell contains a great many respiratory enzymes, reversible redox systems, which are only active at a definite potential. One of the first requirements for a good metabolic functioning of the cell is that the distribution of the potentials in the cell is not disturbed. However, disturbances may result when reducing or oxidising substances are present externally, whose potentials may be carried by permeable redox systems into the cell. For this to happen either the redox systems determining the potential of the medium itself must enter the cell, or some other redox system which can enter the cell and whose normal potential is not too far removed from the potential of the medium must act as a ,,potential carrier''.

When a redox system enters the cell the intracellular potentials will not only be influenced by this external system, but the potential of the latter will be changed as well by the oxidising or reducing properties of the cell contents. The final potential produced in the cell will depend not only on the original potential of the external redox system but also on the poising capacity of this substance in relation to the poising capacities of the intracellular redox systems. This poising capacity of the redox system which enters the cell depends on the concentration of this redox system and on its normal potential where the poising capacity is the most pronounced.

The redox potential of the medium can only influence the development of the cell, when the redox potentials in the cell are changed. In view of the different ways in which the potentials in the cell may be influenced by the potentials of the medium it is not surprising that the relation between the redox potential of the medium and the development of the cell is very complex. Though in general there will be a definite relation between the redox potential of the medium and the development of an organism, this relation may be influenced by different factors: the normal potential of the redox systems establishing the redox potential of the medium, the concentration of these substances, the permeation and the adsorption of the redox systems, other factors of the medium influencing this permeation and adsorption, other factors of the medium influencing the metabolic activities of the cells and so on.

It has not proved possible to obtain growth of aerobic organisms under anaerobic conditions by adding redox systems other than oxygen which establish a high redox potential in the culture solution. This was to be expected. Oxygen not only allows certain metabolic reactions in the cell to proceed by establishing a suitable potential, but itself takes part in these reactions. During the respiration of the cell oxygen acts as an acceptor of electrons and is reduced by the enzymes of the cell. When oxygen is replaced by some other redox system it is not at all certain that this redox system can act in the same way with the respiratory enzymes of the cell. Furthermore, this redox system must be added to the medium in a high concentration to take care of the continuous reduction of the oxidised form by the activity of the living cell. On the other hand the concentration of oxygen in the medium can be relatively small, since the oxygen, when reduced, is continuously replaced by diffusion into the medium from the air. So while the active concentration of oxygen may be very small, the atmosphere acts as an immense oxygen buffer. Finally, the difficulties encountered when trying to replace oxygen by other redox systems may be caused by the specific peculiarities of oxygen with regard to its permeability or specific adsorption.

Summary.

1. Under aerobic conditions the development of *Azotobacter* is suppressed by the presence of reducing substances in concentrations lowering the aerobic potential more than ± 120 mV.

2. The development of *Azotobacter* is not only retarded by the presence of these reducing substances, but the cells are killed.

3. Under anaerobic conditions no development of *Azotobacter* could be observed even when the solution was poised at a high redox potential. The cells were not killed by the anaerobic con-

ditions; as soon as aerobic conditions were restored, a rapid development of *Azotobacter* was observed.

4. When the anaerobic conditions coincide with a low redox potential *e.g.* in culture solutions containing glucose, the cells of *Azotobacter* were killed.

5. The limits of potential endured by *Azotobacter* appeared to be dependent on the relations between the ions in the medium.

6. The cells of *Azotobacter* secrete certain substances enabling them to develop at low potentials.

7. The conclusion is drawn that the development of *Azotobacter* is in general influenced by the redox potential of the medium. However, this influence may be very complex, as the potential of the medium is only important in so far as the redox potential(s) in the cell are changed. The factors influencing the relation between the redox potential of the medium and the redox potentials of the cell are discussed.

8. For *Azotobacter*, oxygen cannot be replaced by other redox systems with a suitable redox potential, most probably because these redox systems have to react with the respiratory enzymes of the cell.

Literature.

1. H. A. ABRAMSON and I. R. TAYLOR, J. Phys. Chem. **40**, 519, 1936. – 2. W. P. ALLYN and I. L. BALDWIN, J. of Bact. **20**, 417, 1930. – 3. W. P. ALLYN and I. L. BALDWIN, J. of Bact, **23**, 369, 1932. – 4. L. W. BROWN and I. L. BALDWIN, J. of Bact. **23**, 56, 1932. – 5. L. W. BROWN and I. L. BALDWIN, J. of Bact. **25**, 29, 1933. – 6. H. BORTELS, A. f. Mikrob. **1**, 334, 1930. – 7. H. BORTELS, Zentralbl. f. Bakt. II, **95**, 193, 1936. – 8. T. Y. KINGMA BOLTJES, Onderzoekingen over nitrificeerende bacteriën. Diss. Delft,1934. – 9. G. KNAYSI and S. R. DUTKY, J. of Bact. **27**, 109, 1934. – 10. I. M. KORR, J. Cell. Comp. Phys. **11**, 233, 1938. – 11. H. PLOTZ et J. GELOSO, Ann. d. l'Inst. Pasteur **45**, 613, 1930. – 12. B. VENNESLAND and M. L. HANKE, J. of Bact. **39**, 139, 1940. – 13. W. B. WOOD, M. L. WOOD and I. L. BALDWIN, J. of Bact. **30**, 593, 1935.

(From the Laboratoria of the State Serum Institute at Rotterdam).

A PECULIAR EXAMPLE OF BACTERIAL ANTAGONISM

by

J. I. TERPSTRA

(Received October 9, 1946).

Some time ago we noted an antagonism between two different species of bacteria. The case was the following:

On an agar plate which had been uniformly seeded with bacteria (*Pasteurella avicida*) and on which the colonies had appeared after 24 hours' incubation a circular area occurred in the centre of which a bacterial colony atypical for oval bacilli had developed: In this area with a radius of 1 cm no further colonies had developed. The central colony was transferred, grown in pure culture and inoculated in dots on plates which a short time earlier had been seeded with oval bacilli. After 24 hours' incubation again, among the regularly occurring colonies of oval bacilli, circular empty spaces could be noted in the centre of which the culture which had been inoculated in dots had developed a colony.

Evidently a phenomenon of antagonism between different microbes was met with here. When investigated more closely the following properties of the inhibitory bacterium were brought to light.

Microscopical properties of an agar culture.

Gram positive coccus. When studied more closely the picture as to size and staining intensity of various forms and their mutual grouping is far from uniform and pictures can be noted in which it is difficult from the forms occurring simultaneously to construe their physiological sequence. The Giemsa preparations served best in this respect. In these the following could be noted: cocci of different size, staining more or less intensively. They occur singly and in groups, tetrades predominate. The single cocci as well as the groups are surrounded by a capsule which shows as a light refracting zone. Evidently the divisions of the cocci are directed perpendicularly one upon the other. The following pictures make this probable: Large somewhat oviform organisms, staining intensively blue, in the centre of which the color may be faded out, suggestive of two cocci. Groups of two cocci in the centre of either of which the color has faded out, however, in a direction perpendicular on that of the preceding division, thus groups of four cocci becoming visible. The separate organism of these groups of four are often smaller than the organisms occurring singly and may be sickle- or rod-shaped. They may more frequently be noted in older than in younger cultures. Preparations from broth cultures do not differ essentially from those from agar cultures.

Cultural characteristics.

In agar cultures fairly small, originally grey translucent colonies which coalesce and develop within 24 hours into a fairly thin pellicle. In broth culture an even slight turbidity with a sediment which whirls upwards in strands when shaken. The growth in serum agar and in serum broth is somewhat more abundant than on agar or in broth.

In bloodagar the nutrient medium under the colony and in its direct neighbourhood is partly clarified.

Growth under aerobic and anaerobic conditions. Growth at a pH of 6.0 and 7.6. Optimal growth at 37° C., possible, however, at room temperature and lower. Killed off after 30′ at 60° C. Gelatine is not liquefied. Indole negative. Litmus milk unchanged. Reduction of nitrate slightly positive. Methyl red test negative. Acid is formed out of glucose, lactose, maltose, glycerol, salicine and saccharose. No changes in inuline, mannitol, trehalose, sorbitol and amygdaline. Catalase slightly positive. No disease could be induced in mice or guinea-pigs by subcutaneous or intraperitoneal injection, neither in rabbits by intravenous injection.

These properties seem to warrant the conclusion that these cocci belong to the genus *Gaffkya*.

For several bacteria their degree of sensitiveness against these inhibitory bacteria (I.B.) was established. Most sensitive were *Pasteurella avicida*, *Bacillus orpheus* and *Staphylococcus*. Sensitive were *Brucella abortus*, *Shigella equilli*, *Bacillus anthracis*, *Corynebacterium pyogenes*. Slightly sensitive to insensitive were *Escherichia coli*, *Salmonella abortus equi*, *Shigella dysenteriae*, *Salmonella typhimurium*, several strains of Streptococci, *Escherichia rhusiopathiae*.

This sensitiveness has been studied on agar plates. This may be carried out in various ways, *e.g.* the agar plate is seeded uniformly with the bacterium to be tested and the I.B. is inoculated in dots on some areas of the same plate. By these means during incubation circular empty spaces arise of a size depending on the sensitiveness of the former bacterium. On the other hand it may be studied whether the growth of the I.B. in its turn is inhibited by another bacterium by seeding the agar surface uniformly with the former and inoculating one of the other bacteria in dots among them. Also some bacilli have been studied in this way. Thus for instance under such conditions a *Staphylococcus* appeared to be able to inhibit the growth of the original I.B. in a small measure and *Bacillus orpheus* did so in a fairly high degree. Tested under definite conditions Staphylococci appeared to be unable to develop within a distance of 16 mm of the I.B., whilst still higher values were obtained with *Bacillus orpheus* and oval bacilli.

By such simple means an impression is gained whether various bacteria influence each other strongly and in which direction. This is a mere question of observation.

Establishing, however, the actual cause of the inhibition is quite another question, which first of all offers theoretical problems. Which should be the leading idea in the starting of the experiments? For the sake of simplification it has been assumed in this investigation that the inhibition of growth is due to a definite chemical substance, thus the function of the bacterium being substituted by the action of a chemical substance. It is clear, however, that this is merely allowed under definite conditions. In fact the problem is put simpler than it actually is. An inhibiting symptom is a result of the interaction of two living organisms, where factors such as pH and redox potential, temperature and nutrition play an important part.

For different bacteria the optima for these factors may lie on quite different levels. Therefore it has been tried to explain the inhibiting symptoms by studying the I.B. in its behaviour towards a definite bacterial species, *e.g.* a *Staphylococcus*. For it is very improbable that the I.B. would modify the pH and redox potential of the medium in such a degree that the *Staphylococcus* would be injured. In fact when, tentitavely the medium has been supplied with an indicator, the color is not altered in a measure indicative of great changes, at all events not in a measure injurious for the Staphylococci. Likewise as for the I.B. the optimal growth of the *Staphylococcus* occurs at 37° C. and it develops well on agar. It is further improbable that the I.B. would draw substances of such a nature from the nutrient medium that the growth of the *Staphylococcus* would be rendered impossible. The correctness of this assumption has been confirmed by later experiments.

The assumption that the inhibition would be caused by a definite chemical substance has still further consequences for the experiment. In fact the action of a substance on the function of a living organism may be growth stimulating or inhibiting whilst also both actions may occur side by side; this depends on the nature as well as on the concentration of the substance. In fact a substance in high concentration may act lethally, in low concentration growth inhibiting and in very low concentration even stimulating. The possibility, however, that the experiment may evidence this, depends on the rate of diffusion of the substance and on the height of the layer of agar. In a deep layer the substance will be diluted in a limited space at a quick rate and it will be hard to distinguish zones where growth is inhibited from those where it is stimulated. Thus it is advisable always to use layers of a same height, which has to be relatively low.

In judging the action of the substance it has to be kept in mind that the result may differ quantitatively as well as qualitatively according to the stage of development of the germs acted upon *viz.*, in repose, dividing or having formed colonies. Either bacterium thus will have to be tested in the various stages and in definite amounts.

Taking these considerations into account finally the experiments were put up as follows:

1. As it has been noted that Staphylococci, inoculated in various ratios against an I.B., were inhibited over a distance of more than 15 mm, it was deemed probable, that the chemical substance would have a considerable rate of diffusion and thus might be filtered off. In order to ascertain this, filtration experiments were run, in which the I.B. was filtered from broth culture through a Seitz filter E.K.; subsequently inhibition tests on agar have been carried out.

The result, however, was very unsatisfactory. Although a few times it might appear that the filtrate would inhibit the Staphylococci, this action was very inconstant and at all events much weaker than that of living bacteria. The filtration tests were repeated several times with broth cultures of various ages — from 5 hours to 10 days — and after various substances had been added, such as serum, peptone in various amounts, some sugars and glycerol. Besides the I.B. were inoculated in a shallow layer of liquid (petri dish with broth) and in other cases I.B. and Staphylococci simultaneously in broth in such a ratio that the former when streaked on an agar plate would have inhibited the latter. As the filtrate of all these cultures could not induce any inhibiting action it was concluded that either the inhibiting substance was difficult to separate from the bacteria in a broth culture or was formed in such minute amounts that it was hard to detect experimentally

2. A 24 hours' agar culture of the inhibitory bacterium is shaken off with some ml of broth. A drop of this broth culture is streaked over the surface of an agar plate. When this streak is dried up, perpendicularly on the thus prepared culture a drop of a *Staphylococcus* culture is streaked. After 24 hours' incubation at 37° C. the I.B. appears to have developed over the total length of the plate, whilst the growth of the Staphylococci has been interrupted over a distance of some 6 mm (Fig. 1).

When this inhibition zone is studied with a pocket lens, it strikes that in the immediate neighbourhood of the I.B. no trace of growth of Staphylococci can be detected whilst at a certain distance of the I.B. in the direction of the non-inhibited colonies of Staphylococci small colonies occur. These colonies vary in size but are all much smaller than normal ones and they occur mostly separately. They are circular and mostly translucent. When these colonies are transferred into broth the smaller ones do not develop, whilst the larger ones do.

a. A Gram preparation of the inhibited colonies of smaller size offers the shadowy picture of cocci, which have generally taken on the stain poorly and are faded, whilst often they have not stained positively.

In the above case the Staphylococci are thus inhibited in growth. The inhibition in the vicinity of the I.B. is the result of a lethal action, starting from the I.B. The inhibition in the neighbourhood of the non-inhibited Staphylococci is the result of a growth inhibition which has left the vitality unimpaired.

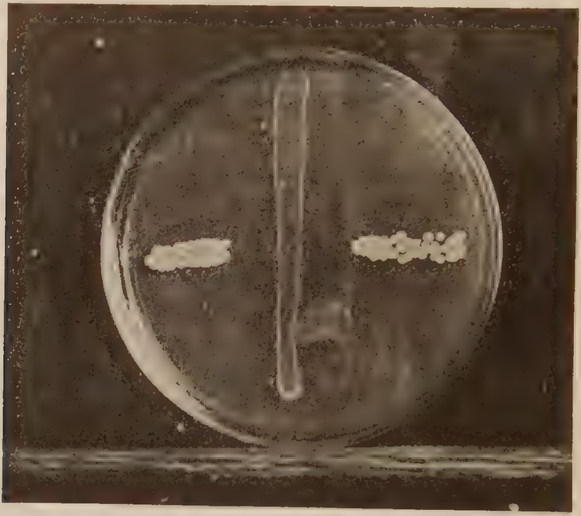


Fig. 1.

b. When the latter experiment is repeated, modifying the ratio of I.B. and Staphylococci, the zone of inhibition appears to increase along with an increase in the amount of the I.B. (up to 16 mm) and to decrease when the amount of Staphylococci is increased, so that finally the Staphylococci — be it in a very small measure — may inhibit the growth of I.B.

c. A definite amount of I.B. is inoculated as under *a* against a definite amount of Staphylococci. The plates are inoculated at 37° C. under anaerobic conditions. The degree of inhibition, measured by the size of the inhibition zone appears to be smaller than when incubated aerobically. In judging this result it has to be kept in mind that the growth of either organism is probably acted upon differently by the change in conditions. Thus without closer investigation the conclusion may not exceed the statement that under anaerobic conditions the inhibiting substance as well is formed.

d. Agar plates of different pH, *e.g.* 6.0 - 6.8 - 7.2 and 7.8 are all inoculated as under *a*. In all those cases the *Staphylococcus* is inhibited. The strongest inhibition occurs at pH 7.2 and 7.6. In what measure this phenomenon may depend on the modified possibilities of growth for the different bacteria failing any further study must be left open.

e. Serum agar and blood agar plates are inoculated as under *a* and incubated at 37° C. Under these conditions the *Staphylococcus* is inhibited in a markedly lower degree. The possibility, however, that the *Staphylococcus* might for its growth draw more advantage from the serum or blood supplied than the I.B. is not excluded here.

3. Various agar plates are streaked as usual with a culture of Staphylococci. One of these plates moreover is inoculated with the I.B., the streak being directed perpendicularly over the former. Then the plate is incubated 24 hours at 37° C. The other plates are incubated directly and are inoculated with the I.B. after 2.5, 5, 7 and 24 hours respectively; after a further 24 hours' incubation they are judged as to inhibition. In the latter cases the *Staphylococcus* has been given the advantage in development of a varying number of hours. It appears that the inhibition zone which for a simultaneous inoculation would have amounted to about 10 mm, after a lapse of 2.5 hours has decreased to 8 mm, of 5 hours to 5 mm and of 7 hours to 2 mm. After a lapse of 24 hours, when the *Staphylococcus* had completely developed, naturally no inhibition zone occurred. From the latter plate, however, a trace of *Staphylococcus* culture was taken on the spot where the I.B. touched the *Staphylococcus*. With this material broth was inoculated. After 24 hours' incubation this culture of Staphylococci appeared to have developed normally.

Thus it may be concluded from this experiment that the I.B. inhibits the growth of *Staphylococcus* merely in a definite stage of development of the latter. When the *Staphylococcus* has developed normal colonies, it is apparently not further acted upon.

4. The I.B. is streaked on a number of agar plates. One of these is streaked directly in the usual way with the *Staphylococcus* and incubated. The other plates are streaked with the *Staphylococcus* and incubated after 1 × 24 hours, 2 × 24 hours and 4 × 24 hours.

The inhibition zone, which at direct inoculation by both organisms and after a 24 hours' advantage of the I.B. amounts to about 10 mm, has decreased on the plate which has been streaked with *Staphylococcus* after 48 hours to 2 mm. Moreover some *Staphylococcus* colonies have developed next to the I.B. colonies already developed on the spot where both species of bacteria coalescence. This is still more distinct on the plate which has been inoculated with the *Staphylococcus* after 72 hours, whilst in the plate kept 96 hours before inoculation the *Staphylococcus* did not suffer any injury from the I.B.

From this and the former experiment it may be concluded that the occurrence of the inhibitory effect depends on the stage of growth of either bacterium. On one hand no influence can be noted when the culture of *Staphylococcus* has already developed completely; on the other hand the I.B. can merely act during its active growth, *e.g.*, in the first 24 or 48 hours after its transfer. In the following the lapse of time during which inhibiting substances are formed is studied more closely.

5. The I.B. is inoculated in the centre of some agar plates by streaking out one drop and incubated during periods of different length (*viz.*, 1, 2, 4, 7, 16, 24 hours, 48 hours and 13 days).

Then the part of the nutrient medium, upon which the I.B. has developed is cut out and a *Staphylococcus* is inoculated on the remaining part of the plate adjacent to the part cut out, whereupon the plate is once more incubated. The occurrence either of inhibition of growth, partial growth or full development was a measure whether the I.B. within a definite period had formed sufficient inhibiting substances. The result was that after a 2 hours' growth of I.B. a partial inhibition might occur, whilst after 5 hours' growth the concentration of the inhibiting substance was such that the Staphylococci were completely inhibited in growth. A same complete inhibition occurred also after 16 hours' growth; whilst often on plates on which the I.B. had grown during 24 hours a marked decrease in inhibition could be noted. On plates having been seeded still earlier any inhibition was mostly quite out of question.

Thus it may be concluded that the formation of inhibiting substance in notable quantities occurs within the initial 24 hours of growth, whilst it has to be assumed that the durability of these substances in the nutrient medium is fairly small. The following experiment aimed at the gaining of an impression of the length of this durability.

6. Agar plates were inoculated with I.B. as under 5 and incubated and cut out likewise. This time, however, they were not inoculated directly with *Staphylococcus* but after a varying lapse of time. It appeared that in some cases after 3 hours, in other cases, however, not even after 8 hours a decrease in the inhibiting effect could be noted. Plates which had been seeded with *Staphylococcus* directly after the cutting out of the I.B. served as controls.

Whereupon depends this relative indurability of the inhibiting substances? Apparently two possibilities might be assumed: inactivation or evaporation of the inhibiting substance. As far as known to us inhibiting substances which may be detected in a nutrient medium as mentioned and are volatile as well, have not been described up till now. Notwithstanding this some experiments have been put up which might point to the existence of a certain volatility.

7. A drop of culture of Staphylococci is streaked in the usual way on the agar plate. On either side of this streak, part of a slide is placed. A piece of agar which has been inoculated about 5 hours earlier with I.B. is placed over the culture of Staphylococci in such a way, that its ends rest on the slides. Both species of bacteria are thus separated by a thin layer of air and the layer of agar seeded with the

I.B. The plate is incubated at 37° C. After 24 hours the development of *Staphylococcus* appeared to be interrupted in the region exactly below the piece of agar inoculated with the I.B. When this experiment was repeated with freshly inoculated pieces of agar, hardly ever any inhibition could be noted. Inhibition, however, could actually be found, when the nutrient medium had been seeded with I.B. 5 hours before the pieces of agar inoculated with *Staphylococcus* were placed over it. This, however, succeeded merely when the seeded surface of the piece of agar was directed towards the culture of I.B.

One is apt to explain these symptoms by assuming that the substances formed by the I.B. volatilize during or after diffusion in the agar medium and in such a concentration that they may inhibit the growth of *Staphylococcus* at a small distance.

Two symptoms, however, occurred in these inhibition tests, which were studied more closely.

a. In the whole series of tests it was especially at the start that the regions of inhibition were sharply limited. The spot where the Staphylococci were completely inhibited bordered abruptly on a zone of uninhibited growth. It was remarkable that in the experiment in which the agar bridge had been seeded with I.B. its form induced the shape of the inhibited region in the petri dish. Thus the impression was gained that the inhibition would proceed in a definite direction.

b. When the height of the layer of air between *Staphylococcus* and I.B. had been increased by means of placing a second slide on the top of the first, no inhibition of growth could be noted.

So the inhibition through the air seemed to be connected with a definite minimum distance.

It was studied in how far symptoms *a* and *b* were actually significant and might agree with the properties of a volatile substance.

8. The experiment was put up as under 7 with two agar plates. In one of them, however, by special device disturbance of the air was brought about. It was expected that along with the air the volatile substances would be transported and the inhibition would no longer be sharply directed. The result, however, offered no change in the nature of the inhibition; the direction of the inhibition was unchanged when compared with the control.

In judging this negative result the possibility has to be kept in mind that normally the ventilation in a petri dish might be such that its modification might not bring about any change in the inhibitory effect. Thus this experiment was not repeated. Moreover later experiments showed that directed inhibition might tally with the presence of a volatile substance and that under influence of certain conditions inhibition in definite direction might got lost.

In fact when these experiments were repeated after a month, the inhibitory effect could no longer be induced, whilst the experimental conditions apparently had remained the same. It was not deemed probable that this would be caused by some change in the biochemical behaviour of the I.B. as numerous subcultures originated from earlier cultures could neither induce the effect. Moreover inhibition in the nutrient medium where both species of bacteria had been inoculated side by side or over each other, occurred in a same measure as formerly. The cause of this change in behaviour, however, was looked for in a change in the nutrient medium set in meanwhile, which had been left unnoticed, the more so as a fresh batch of nutrient agar had been used. When the test was repeated on nutrient agar derived from other laboratories the inhibiting effect appeared to occur again on some media and in several cases even much more strongly than originally. In the latter cases the extension was such that no connection could be traced with the shape of the piece of agar applied; thus no definite direction of the inhibition appeared to occur (Fig. 2).

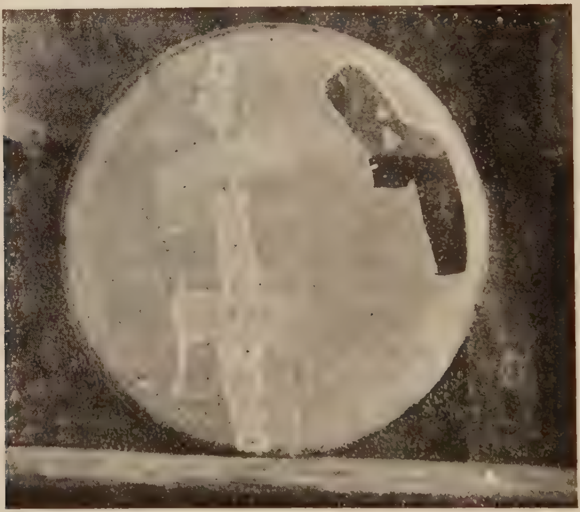


Fig. 2.

The connection of the inhibition through the air with a definite minimum distance neither appeared of essential significance as may be concluded from the following.

9. Some watch glasses are filled for the greater part with agar and inoculated with the I.B. and incubated during 5—10 hours. *Staphylococcus* is streaked on large agar plates and the watch glasses with the seeded surface directed towards the *Staphylococcus* cultures are placed on the agar plate and shut off air tight by means of paraffine. By these means the Staphylococci in a narrowly limited space are at a distance from the I.B. slightly exceeding the thickness of a slide. In a few cases the Staphylococci after

incubation appeared not to have developed in contradistinction to control cultures put up simultaneously. So it was shown that the inhibiting effect through air may be induced over a wider distance when any ventilation, *e.g.* a decrease of the concentration of the inhibiting substances, has been prevented.

The fact that the inhibiting effect, transmitted through the gas phase under anaerobic conditions often may be induced over a wider distance than aerobically agrees with the above.

The latter tests point very strongly to the presence of a volatile substance and thus the fact that inhibiting substances fairly soon after their origin can no longer be detected in the nutrient medium, may be ascribed to this property.

Antibiotica which may be detected by their diffusion in the medium as well as by their volatibility outside the medium apparently have not been described thus far. In the summarizing work of WAKSMAN on antibiotica (1945) no example of such symptoms is apparently offered. Thus it would be of value to know the exact conditions needed for the induction of this symptom. It is clear that these are not completely known as yet. Nutrient media seemingly of the same composition offered different results as soon as inhibition induced at distance was aimed at.

Initially the cause was looked for in a difference in the heat of sterilization and in fact a stronger heating of the agar medium appeared sometimes to favour the inhibitory effect through the air. This was, however, by far not always the case. Besides it appeared that in various parts of a same plate inhibitions of various strength might be induced. Moreover, in how far the condition of the I.B. has to be taken into account for its explanation and whether eventual currents of air may play a part, are questions hard to answer.

But next to these unanswered questions the remarkable fact holds good that in many well-controlled cases an inhibiting symptom between two species of bacteria which were not in mutual contact by means of the nutrient medium could be noted. The assumption that volutatile substances, formed by one or either of these species will induce this phenomena, seems the only conceivable one.

Summary.

An example of antagonism has been described. The properties of the growth inhibiting bacterium have been investigated more closely. These appear to agree nearly fully with those of the genus *Gaffkya*.

The development of *Pasteurella avicida, Bacillus orpheus* and *Staphylococcus* was strongly inhibited by this bacterium. Other species are less sensitive or insensitive.

After the theoretical problems have been discussed, which such

investigations give rise to, it has been studied under which conditions Staphylococci are inhibited.

The occurrence of the inhibition effect depends on the stage of development either bacterium is in; *Staphylococcus* having developed completely it will not notably be acted upon. Initially inhibition of growth occurs along with dying off of *Staphylococcus*. In a somewhat later stage the vitality remains unimpaired.

The inhibiting bacteria produce inhibiting substances merely in the first 24 hours. In the filtrate of a broth culture these are hard to detect. Their behaviour in agar plates makes it apparent that their action depends strongly on the concentration of the antagonist.

The durability of the inhibiting substances in the agar medium is not great. In all probability this depends on a volatibility of these substances.

(From the Bacteriological Laboratory of Lever Brothers and Unilever N.V.
Rotterdam).

A REACTION ON THE FORMATION OF HYDROGEN PEROXIDE BY MICROORGANISMS IN SOLID MEDIA

by

A. KNETEMAN

(Received November 11, 1946).

SÖHNGEN (9), in a publication on changes manganese compounds undergo by microbiological processes, described the dissolution of manganese dioxide in agar plates by bacteria growing on the surface. The black precipitate obtained when mixing solutions of manganous sulfate and of potassium permanganate was used by SÖHNGEN in these experiments.

Several microbes appeared to be able to dissolve this preparation of manganese peroxide present in the nutrient medium, such as: sporeforming aerobes, coli bacteria, yeasts, lactic acid bacteria, *Oidium lactis*, etc.

This reaction was attributed by SÖHNGEN to the formation of organic oxy-acids from glucose by the organisms as it was found that when small quantities of pure oxy-acids: lactic acid, citric acid, gluconic acid, tartaric acid, malic acid, etc. were put on the surface of such agar plates, the black oxide was dissolved leaving transparent fields. Other acids such as fatty acids, succinic acid and others not belonging to the oxy-acids and also the mineral acids phosphoric and sulfuric acid did not dissolve the peroxide. There was one exception *viz.*, oxalic acid; although being no oxy-acid it readily dissolved the MnO_2.

When repeating these experiments, the results obtained by SÖHNGEN could be confirmed. However, we observed a considerable difference as regards the action of the pure acids compared with the action of bacteria. The latter dissolved the dioxide far more vigorously than did the pure oxy-acids. The transparent fields around the colonies sometimes were so large that it became very improbable that these were caused solely by the oxy-acids formed.

We further found that when using the mineral „Pyrolusite" in finely powdered form (henceforth indicated in this publication as m.d.o.) instead of the manganese dioxide obtained by precipitation as used by SÖHNGEN in the culture plates, this showed a distinctly different behaviour. The mineral m.d.o. was found to be dissolved by a comparatively small number of different bacteria species. They all belonged to the group of lactic acid bacteria and allied micrococci and streptococci. The bacteria mentioned by SÖHNGEN as positive on his plates, *viz.*, sporeformers, *B.coli*, *B.aerogenes*, yeasts, *Ps.fluorescens*, etc. did not dissolve this m.d.o.

Further it was found that certain moulds also were able to dissolve the mineral m.d.o. in the plates.

Contrary to SÖHNGEN's observations most of the organic acids mentioned before did not dissolve this form of manganese dioxide. Among the acids tested only oxalic acid, ascorbic acid, citric acid and pyruvic acid appeared to be able to dissolve the m.d.o. Of these acids oxalic acid and ascorbic acid dissolve the m.d.o. most vigorously. Small quantities of these acids when brought on a m.d.o. plate give considerable transparent fields even in the cold (± 15° C.); citric acid does not react at all at this temperature not even if quantities of 200 mg are brought on the plates.

However, at 30° C. a distinct dissolving of m.d.o. is noticed already with quantities of 10 mg citric acid. Pyruvic acid has only a very weak m.d.o. dissolving capacity; like citric acid it does not act at 15° C. but at 30° C. a small transparent field was obtained if larger quantities of the acid were used. As the latter four acids are certainly not produced in noticeable quantities by the bacteria involved in these experiments the phenomenon evidently must have another cause. The agent responsible for this reaction was found to be nothing else but hydrogen peroxide which combined with the acid produced simultaneously by the microorganisms dissolves the m.d.o.

In the course of investigations carried out some fifteen years ago on the influence of oxygen on the metabolic processes of lactic acid bacteria it was observed that considerable amounts of hydrogen peroxide can be produced and that the injurious effect of the accumulation of H_2O_2 could be neutralised by the addition of finely powdered pyrolusite.

It is evident that the dissolution of the m.d.o. in culture media by bacteria must also be due to the formation of hydrogen peroxide and acid. Whilst in culture plates prepared with the mineral pyrolusite only H_2O_2 is responsible for the reaction, in agar media prepared with the precipitated MnO_2 as used by SÖHNGEN besides the organic oxy-acids also the H_2O_2 in certain cases will play a part.

PREPARATION OF THE M.D.O. MEDIUM.

The m.d.o. medium is prepared as follows:

As a basal medium meatbroth agar with the addition of 5 % yeast autolysate (10 % solids) and 1 % glucose is used. Finely powdered pyrolusite in a 40 % suspension in water is autoclaved and kept ready for use in small plugged flasks. In petridishes of 10 cm diameter about 20 cc of the basal medium is poured and after cooling and solidification a thin layer of the same agarmixed thoroughly with 4—5 drops of the m.d.o. suspension is poured on top of the first layer. The amount of m.d.o. suspension is adjusted in such a way that the plate has a black appearance but is distinctly transparent. The basal medium can be changed when required; *e.g.* for the lactic acid bacteria in sour milk, starters, etc. ordinary whey agar to which after cooling down to ± 50° C. sterile milk is added can be used advantageously.

In the experiments with Söhngen's manganese peroxide the first mentioned basal medium was used. The precipitate obtained by mixing solutions of manganous sulfate and permanganate after washing was suspended in water and autoclaved. The plates were made in the same way.

In order to make quite sure as to the nature of the reaction on the culture plates described above and to prove that H_2O_2 is indeed the principal reagent we carried out the following experiments. For comparison we have made use also of the well-known blood benzidine agar.

Experiment I.

Several strains belonging to the group of lactic acid bacteria were tested by making streaks on m.d.o. plates as well as on Penfolds blood benzidine agar (7). The blood plates were prepared with the same basal medium without glucose by adding 5 to 10 % of a solution of 0.25 % benzidine hydrochloride and 5 % fresh cow's blood. The plates were incubated at 30° C. The result was that the strains which dissolved the m.d.o. also gave the well-known blueish-black coloration on the blood agar and none of the strains that failed to dissolve the m.d.o. reacted on the blood plates. This uniformity in reaction strongly suggests that in both cases the same reagent is active.

Experiment II.

Bacteria which dissolved the m.d.o. in the presence of air failed to do so when the plates were incubated in an anaerobic jar in a hydrogen atmosphere and also the bloodplates showed no reaction under these circumstances, although on both kinds of plates growth was abundant. If the same experiment was carried out with Söhngen's peroxide this was found to be dissolved slightly, also by strains which did not give a reaction on m.d.o. or bloodplates in the presence of air. This must be ascribed to the formation of lactic acid (oxy-acid) which is not prevented by the anaerobic conditions. It must be emphasized, however, that the transparent fields are much larger when the plates made with the precipitated peroxide are exposed to the air.

This experiment shows clearly that for the reaction on the m.d.o. and the blood benzidine plates the presence of oxygen is a necessary condition.

Experiment III.

The following experiment shows that the dissolving of m.d.o. in the solid medium and the reaction on the blood plates do not

take place as soon as the enzyme catalase is present. This could be demonstrated convincingly with the aid of special agar plates consisting of the following four layers of agar on top of each other.
1. plain basal medium,
2. the same medium with added m.d.o.,
3. basal medium containing an active preparation of catalase,
4. a thin layer of basal medium.

Plates of this composition inoculated with streaks of m.d.o. dissolving lactic acid bacteria did not show the reaction at all, which obviously must be due to the fact that the hydrogen peroxide formed by the bacteria on the surface of layer nr. 4 was decomposed by.the catalase in layer 3 before it could reach the m.d.o. in layer 2. The reaction, however, became visible when the plates were kept in the incubator for a long time (a week). This was caused by the excess of acid formed in the medium which either destroyed or inactivated the catalase. Thus the effect of the catalase is only striking as long as it is active and the experiment was very convincing when for comparison a similar plate was inoculated in which the catalase beforehand had been destroyed by heating.

In the experiment carried out with the peroxide obtained by precipitation also a slight dissolution was observed as in the previous experiment with the exclusion of air and which is due to the formation of lactic acid. On blood benzidine plates covered in the same way with a layer of agar containing catalase no reaction was visible when inoculated with the same strains of lactic acid bacteria.

The catalase preparation used for this purpose was made by treating a strong suspension of a catalase producing micrococcus with alcohol, separating the dead bacteria from the liquid by centrifuging, drying the bacteria on sterile filter paper and suspending them in sterile water. The prevention of these reactions by the presence of catalase — the specific enzyme which in living organisms, tissues, etc. decomposes the poisonous H_2O_2 formed -- leaves no doubt that the principal reagent must be hydrogen peroxide. Moreover the following observations give additional proof.

It was often found that under the streaks of strains which dissolve the m.d.o. most vigorously gasbubbles were formed in the agar, especially when the plate was covered by a layer of medium containing no m.d.o. It need not be proved that the gas developed can be no other than oxygen which through the presence of an additional layer of agar is prevented to evaporate. If the m.d.o. is omitted from the agar and lactic acid bacteria have developed on it, H_2O_2 often can be detected by flooding the plate with SCHÖNBEIN's reagent.

Finally, as already said in the beginning of this paper, drops of H_2O_2 together with some acid — be it lactic acid or sulfuric acid — readily dissolve the m.d.o. in the plates, whilst drops of acids alone do not (with the exception of oxalic, ascorbic and citric acid).

From the observations mentioned above we conclude that the dissolving of m.d.o. in the culture plates of the composition described without any doubt must be ascribed to the action of hydrogen peroxide formed by the microbes. Consequently this medium can be successfully used for the detection of H_2O_2-producing bacteria.

The possibility that in our medium the m.d.o. is dissolved by the action of hydrogen donators, the m.d.o thus acting as hydrogen acceptor as was observed by MANN and QUASTEL (4) can be left out of consideration as in that case the reaction would also take place in an atmosphere free from oxygen. It is even possible that in MANN and QUASTEL's experiments the transformation of manganese dioxide to manganous compounds in soils will be partly due to the formation of H_2O_2. This transformation took place only in the presence of glucose, and oxygen from the air not being excluded, the conditions for the formation of acid and H_2O_2 are realised.

REACTION OF THE MEDIUM.

The m.d.o. medium before inoculation has a neutral reaction but as m.d.o. dissolves far better under the influence of H_2O_2 in acid medium, it is essential that glucose is present. Hence the medium is especially suited for the detection of H_2O_2 formed by those bacteria, *e.g.* the lactic acid bacteria, that produce acid from glucose and form no catalase. The addition of glucose, besides, has the advantage that the production of H_2O_2 is greatly enhanced. For the same reason it appeared to be a considerable improvement to add glucose to the blood benzidine agar. In the first place — as already mentioned — the production of H_2O_2 is greatly accelerated and in the second place the addition of glucose will impart an acid reaction to the medium, which acidity will stop the activity of the catalase present in this medium and deriving from the blood. It is contradictory that a medium like PENFOLD's containing blood catalase would be suitable for the detection of H_2O_2 formed by bacteria. An accurate observation of the reaction on these plates shows that in fact the medium itself does not acquire the dark colour but the dark coloration is limited to the colony. This can be explained by the fact that the blood pigment diffuses into the colony and there it reacts with H_2O_2 formed — catalase being absent. However, H_2O_2 diffusing into the agar will for the greater part be decomposed by the blood catalase present in the agar and consequently around the colony no reaction takes place. If on the other hand glucose is present in the medium a strong dark discoloration is also obtained in the agar as H_2O_2 diffusing into the agar is not decomposed, the catalase being inactivated by the acid reaction. In accordance with this is the fact that a drop of 3 % H_2O_2 put on a PENFOLD blood benzidine agar gives practically no reaction, but a drop of H_2O_2 mixed with some acid gives a strong reaction which must be due to the inactivation of catalase.

SOME REMARKS REGARDING THE USE OF M.D.O. PLATES.

It has been stated before in this paper that bacteria which dissolve m.d.o. also give a positive reaction on blood benzidine agar.

The lactic acid Streptococci from sour milk, however, give a positive reaction on d.m.o. plates but no or only a very slight reaction on blood agar even when it contains glucose or lactose as a source for acid production. This is due to the fact that these strains of lactic acid bacteria produce only little H_2O_2 and consequently the m.d.o. medium is to be considered as far more sensitive for the detection of traces of H_2O_2.

On the other hand strong reactions are obtained with the lactic acid bacteria as are met with in the industry of meat products and which are responsible for the discoloration of meats (see JENSEN (2)). Those non lactose fermenting bacteria produce considerable quantities of H_2O_2 and also give a marked reaction on blood agar but in our opinion the m.d.o. medium in this case is also to be preferred. The addition of m.d.o. to culture media has the advantage that the agar does not become toxic through the H_2O_2 formed and on account of this growth is greatly favoured and goes on for a longer time.

For the preparation of stock cultures of lactic acid bacteria on slopes therefore, it can be recommended to add m.d.o. to the agar as this prevents an early death of the bacteria by H_2O_2 poisoning. It is advisable not to add calciumcarbonate to the medium as this neutralises the acid formed from glucose and consequently the rate of decomposition of H_2O_2 by m.d.o. is diminished. In fact H_2O_2 is far more poisonous for these bacteria than the acid. A too high acidity can be prevented by reducing the amount of glucose in the medium. Is is essential to suspend the m.d.o. equally in the agar by rolling the tubes and covering them with ice. Finally we remark that it must be looked upon as an acquisition to have the disposition of a special culture medium for the detection of small quantities of H_2O_2 instead of the blood benzidine agar generally used for the purpose. Fresh sterile blood in many laboratories is difficult to obtain whereas manganese dioxide is always ready to hand.

FORMATION OF HYDROGEN PEROXIDE BY MOULDS.

Several strains of moulds, mostly *Penicillium* species, have been isolated with the aid of m.d.o. plates and they showed the same reaction as the H_2O_2 producing bacteria. As the mycelium of these moulds contained an abundance of catalase it was supposed that the dissolving of the m.d.o. in these cases was caused by either oxalic or citric acid formed from the glucose in the agar.

It could be demonstrated that a number of these moulds indeed produced a considerable amount of oxalic or citric acid; however, for several strains the production of these acids could not be demonstrated. It is not yet clear to which reaction the dissolution of m.d.o. must be ascribed in this case. It is possible that the reaction is due to H_2O_2 formed in the medium indirectly through the action of the enzyme ,,glucose oxidase'', formed by the mould and diffusing into the medium, where it cataly ses theoxidation of glucose by atmospheric oxygen, thereby producing H_2O_2.

Probably the same enzyme has been found in cultures of *Aspergillus niger* by RACIBORSKI (8) who called it "iodide oxidase" on account of the formation of iodine from iodides when added to the culture liquid. KORCZEWSKI (3) demonstrated the presence of H_2O_2 in the medium and PEARCE (6) revealed the true nature of the enzyme showing that the formation of iodine was due to a secondary reaction between iodide and H_2O_2, the product of the activity of the primary oxidation system. The enzyme responsible for the H_2O_2 formation is identical with the ,,glucose oxidase'' described by MÜLLER (5) and according to COULTHARD *et al.* (1) "Notatin" has the same enzymic properties.

Several of the moulds isolated by us as m.d.o. positive showed a strong inhibitory effect on the growth of sensitive bacteria such as *Staphylococcus aureus*. When pieces of the agar on which the mould had developed were placed on a culture plate in which these bacteria were suspended large blank fields were observed. For several strains this inhibition took only place in the presence of glucose in which cases the inhibition may be due to the formation of H_2O_2 by glucose oxidase.

When exposing m.d.o. plates to the air the m.d.o. positive strains can easily be isolated. However, the number of positive strains is but small; among hundreds of colonies developing on such plates only a few are m.d.o. positive. The plates can also be used advantageously for the isolation of strains of *Asp. niger* which produce much citric acid, these strains giving the largest transparent fields. In this case it is advisable to use cane sugar in the plates instead of glucose or malt extract.

Summary.

In this paper a special culture medium for the detection of H_2O_2 formed by bacteria producing acid from glucose and by certain moulds is discussed and the preparation of the same is described. This medium can be used with success instead of the blood benzidine medium and was found to be decidedly more sensitive in so far that it allows to trace very small quantities of H_2O_2. The mineral manganese peroxide as used in this medium differs in many respects from the precipitate obtained by mixing solutions of manganous sulfate and permanganate as used by SÖHNGEN.

Experiments with moulds have given indications as regards the production of a soluble glucose oxidase.

References.

1. C. E. Coulthard *et al.*, Biochem. J. **39**, 24, 1945. – 2. L. B. Jensen, Bacteriology of Meats 1945, p. 43. – 3. M. Korczewski, Trans. Polish Acad. Sci. (Cracow) **61**, 29, 1922. – 4. P. J. G. Mann and J. H. Quastel, Nature **158**, 154, 1946. – 5. D. Müller, Biochem. Z. **199**, 136, 1928; **205**, 111, 1929; **213**, 211, 1929; **232**, 423, 1931. – 6. A. A. Pearce, Biochem. J. **34**, 1493, 1940. – 7. W. J. Penfold, Med. Journ. of Australia 1922. – 8. M. Raciborski, Bull. Acad. Sci. Cracovie 693, 1905. – N. L. Söhngen, Centralbl. f. Bakt. II, **40**, 545, 1914.

(From the „Rijks Instituut voor de Volksgezondheid" at Utrecht).

STUDIES ON STAPHYLOCOCCI

I. THE PLASMA COAGULATION REACTION

by

J. H. BEKKER

(Received December 24, 1946).

It is often difficult to ascertain whether an isolated *Staphylococcus* is of pathogenic significance. As a rule when a pure *Staphylococcus aureus* strain is isolated from a pathological process the etiological relation to the disease is clear, but the ubiquity of staphylococci infers that these microbes may also be found in pathological processes where they have not necessarily played an etiological role. This uncertainty is accentuated by the fact that the pathogenic characteristics of staphylococci are not definite and that the distinction between pathogenic and non-pathogenic staphylococci is not sharp.

There are many methods for determining the pathogenicity of a staphylococcus strain; pigment formation, hemolysin production, fermentation of mannitol, liquefaction of gelatin, crystal violet reaction, toxin production, etc. None of these methods are found entirely reliable.

Loeb (31), Much (34), Kleinschmidt (29), von Gonzenbach and Uemara (23) found that staphylococci have the property of producing a substance, staphylocoagulase, which is able to coagulate plasma. Von Darányi (12, 13), Grosz (24, 25, 26, 27), Kemkes (28), Gengou (19, 20) a.o. observed that this property was possessed by some albus as well as aureus strains. In recent years much evidence has been obtained that the ability to produce coagulase was found only in pathogenic staphylococci and that this test was the most reliable biochemical reaction for pathogenicity (a.o. Chapman et al. (3, 4, 5, 6), Fisher (16), Cruickshank (11), Neter (35), Cowan (10), Flaum (18), Gillespie, Devenish and Cowan (22), Christie and Keogh (7), Fairbrother (15), Lieb and Nothhacksberger (30), Bøe (2), Sulamaa (38)). Le Cosquino de Bussy, van Loghem and Visser (9) used this test in their study of the occurrence of *Staphylococcus aureus* in the nose of healthy persons.

To investigate the suitability of the plasma coagulation reaction for the differentiation of pathogenic and non-pathogenic strains of staphylococci in our routine work, 250 *Staphylococcus* strains were isolated from different sources (blood, pus, urin, sputum, cerebro spinal fluid, skin, etc.) The isolated strains were tested for pigment formation, hemolysin production, plasma coagulation, toxin production and the clumping in solutions of fibrinogen.

The pigment formation was studied on agar and serumagar plates after 24 hours incubation at 37° C. and thereafter at room temperature for 10 days. The hemolysin production was determined after 24 hours incubation on 5 % sheepblood agarplates. Though the hemolysin formation in bloodbroth perhaps may furnish more accurate results only the routine plate method was used. Different methods for determining the coagulation of plasma are described (a.o. FISHER (16), MACFARLAN (32), DI-ROCCO and FULTON (14), FISK (17), GILLESPIE (21), PENFOLD (36), COLBECK and PROOM (8), WILLIAMS and HARPER (40)). The following technique was used in my experiments: 10 ml of rabbit blood obtained by cardiac puncture were added to 10 ml of 0.4 % sodium citrate in distilled water. The mixture was centrifuged and the supernatant plasma withdrawn. To 0.5 ml of this plasma 10 fold diluted with normal saline were added five drops from a capillary pipette (approximately 0.1 ml) of an overnight broth of a *Staphylococcus* culture. The tubes were incubated at 37° C. and examined after 1, 4, and 24 hours. The undiluted plasma remains suitable for the test for several weeks if kept in the ice-chest.

BERGER (1) described the clumping of staphylococci in fibrinogen solution as a rapid method for the identification of coagulase positive staphylococci. The test is performed by rubbing a loopful of an overnight agarculture in a drop of the fibrinogen solution on a slide. Coagulase-positive strains show a clumping resembling agglutination whereas coagulase-negative strains give a homogenous suspension. The fibrinogen solution is prepared by adding a saturated solution of sodium chloride to an equal volume of citrated plasma; the mixture is kept in the ice-chest for 4 hours. The precipitated fibrinogen is separated by centrifuging and redissolved in a quantity of distilled water equal to the original volume of plasma. This fibrinogen solution, when stored in the ice-chest, can be used for at least 4 weeks.

Next to these biochemical reactions an animal test *viz.*, the production of necrotoxin by staphylococci was performed. 0.1 ml of an overnight broth culture was injected intradermally into guinea-pigs (TIMMERMAN (39)). Readings were made after 24 and 48 hours.

Among the 250 strains studied there was complete agreement in 228 strains (91.2 per cent). Considering the clumping reaction in fibrinogen solution not as a separate reaction but as a result of the coagulating property a deviation of the results was found in 22 strains:

12 strains were hemolysis-negative;
5 strains were pigment-negative;
2 strains were coagulase-negative;

1 strain was necrotoxic-negative;
1 strain was coagulase- and necrotoxic-negative; and
1 strain was pigment- and necrotoxic-negative.

Of the 156 staphylococcus strains where one or more of the investigated properties were positive the necrotoxic activity was positive in 153 strains (98.1 per cent); the coagulation reaction in 152 strains (97.6 per cent), the pigment formation in 149 strains (95.5 per cent) and the hemolysin production in 142 strains (91.0 per cent). Among the biochemical reactions the plasma coagulation test also in my experiments gave the best results.

Table I shows the mutual relation of the reactions studied.

Tabel I

		Pigment		Hemolysis		Coagulation		Clumping in fibrinogen		Necrotoxin	
		+	—	+	—	+	—	+	—	+	—
No. of strains		149	101	143	107	152	98	149	101	153	97
pigment	+			137	12	146	3	145	4	147	2
	—			6	95	6	95	4	97	6	95
hemolysis	+					140	3	137	6	141	3
	—					12	95	12	95	12	94
coagulase	+							149	3	151	2
	—							0	98	2	95
clumping in fibrinogen	+									150	0
	—									3	97

The clumping in fibrinogen solution appeared to be a good preliminary test for the coagulase production. In all cases with a positive clumping test the coagulation was also positive; when clumping is negative a coagulation test must be performed. The majority of the coagulase positive strains were aureus strains (146) but also 6 coagulase-positive albus strains were encountered. We shall not go into the question whether these albus strains were mutilated aureus-strains, which have lost their pigment forming properties (Pinner and Voldrich (37)).

From my experiments it could also be taken that the localisation a staphylococcus strain was isolated from did not determine, whether or not it was pathogenic. Staphylococci with the characteristics ascribed to the pathogenic forms could be found on normal skin and in the nose and other mucous membranes of healthy persons (see a.o. le Cosquino de Bussy, van Loghem and Visser (9), MacFarlan (32), Miles, Williams and Clayton-Cooper (3), Bøe (2)).

When do we regard an isolated staphylococcus strain as pathogenic? According to most investigators pigment-negative, hemolysis-negative, coagulase-negative, etc. staphylococci are slightly or not pathogenic. Pathogenic staphylococci are described as coagulase-positive, usually producing aureus pigment, forming hemolysins, producing toxins, and being virulent for rabbits and guinea-pigs. This view is substantiated by my experiments. For the differentiation between pathogenic and non-pathogenic staphylococci a single biochemical reaction proved not to be reliable. Several tests must be performed for determining the pathogenicity of an isolated *Staphylococcus* strain.

Summary.

The plasma coagulation reaction of staphylococci is discussed. Among the biochemical reactions in use for determining the pathogenicity of staphylococci, this reaction is probably the most reliable.

References.

1. F. M. Berger, J. Path. Bact. **55**, 435, 1943. – 2. J. Bøe, Acta path. microb. scand. **21**, 721, 1944. – 3. G. H. Chapman, et al., J. Bact. **28**, 343, 1934. – 4. G. H. Chapman et al., J. Bact. **33**, 646, 1937. – 5. G. H. Chapman et al., J. Bact. **35**, 311, 1938. – 6. G. H. Chapman et al., J. Bact. **41**, 431, 1941. – 7. R. Christie and E. V. Keogh, J. Path. Bact. **51**, 189, 1940. – 8. J. C. Colbeck and H. Proom, Brit. med. J. **2**, 471, 1944. – 9. I. J. Le Cosquino de Bussy, J. J. van Loghem en A. K. Visser, Ned. Tijdschr. Geneesk. **86**, 1273, 1942. – 10. S. T. Cowan, J. Path. Bact. **46**, 31, 1938. – 11. R. Cruickshank, J. Path. Bact. **45**, 295, 1937. – 12. J. von Darányi, Zentralbl. f. Bakt. I, **99**, 701, 1926. – 13. J. von Darányi, Zentralbl. f. Bakt. I, **134**, 13, 1935. – 14. F. W. Di-Rocco and M. Fulton, J. Bact. **37**, 657, 1939. – 15. R. W. Fairbrother, J. Path. Bact. **50**, 83, 1940. – 16. A. M. Fisher, Bull. Johns Hopk. Hosp. **39**, 393, 415, 1936. – 17. A. Fisk, Brit. J. exp. Path. **21**, 311, 1940. – 18. A. Flaum, Acta path. microb. scand. suppl. XXXV, 1938. – 19. O. Gengou, Ann. Inst. Pasteur **51**, 14, 1933. – 20. O. Gengou, Ann. Inst. Pasteur **54**, 428, 1935. – 21. E. H. Gillespie, Monthly Bull. Min. of Health **2**, 19, 1943. – 22. E. H. Gillespie, E. A. Devenish and S. T. Cowan, Lancet **2**, 870, 1939. – 23. W. von Gonzenbach and H. Uemara, Zentralbl. f. Bakt. I, **78**, 97, 1916. – 24. H. Grosz, Klin. Wschr. **6**, 2281, 1927. – 25. H. Grosz, Klin. Wschr. **12**, 304, 1933. – 26. H. Grosz, Zentralbl. f. Bakt. I, **107**, 198, 1928. – 27. H. Grosz, Zentralbl. f. Bakt. I, **122**, 354, 1931. – 28. B. Kemkes, Zentralbl. f. Bakt. I, **109**, 11, 1928. – 29. H. Kleinschmidt, Z. Imm. Forsch. **3**, 516, 1909. – 30. F. Lieb und W. Nothhacksberger, Arch. Hyg. **131**, 31, 1942. – 31. L. Loeb, J. exp. Med. **10**, 407, 1903-1904. – 32. A. M. Macfarlan, Brit. med. J. 2, 939, 1938. – 33. A. A. Miles, R. E. O. Williams and B. Clayton-Cooper, J. Path. Bact. **56**, 513, 1944. –34. H. Much, Biochem. Z. **14**, 143, 1908. – 35. E. Neter, J. Bact. **34**, 243, 1937. – 36. J. B. Penfold, J. Path. Bact. **56**, 247, 1944. – 37. M. Pinner and M. Voldrich, J. inf. Dis. **50**, 185, 1932. – 38. M. Sulamaa, Acta chirurg. scand. **94**, 291, 1946. – 39. W. Aeg. Timmerman, Antonie van Leeuwenhoek **8**, 41, 1942. – 40. R. E. O. Williams and G. J. Harper, Brit. J. exp. Path. **27**, 78, 1946.

(From the Microbiological Laboratory of the Agricultural University, Wageningen).

THE CAUSE OF THE EARLY GAS DEFECT IN CHEESE AND THE MEANS TO PREVENT IT

by

TH. E. GALESLOOT

(Received January 1, 1947).

Newly made cheese contains lactose, which is converted in lactic acid by lactic acid bacteria, a process which is normally concluded within 24 hours. Next to this lactic acid fermentation the lactose may also gave rise to a fermentation which is coupled with production of gas. This ,,early gas defect'' must be distinguished from ,,late gas defect''. The latter occurs in cheese, having been stored for some weeks, in which the lactic acid is partly converted into gaseous products. So the difference is not merely based on the moment of onset, but is narrowly connected with the nature of the fermentation.

The early gas defect, causing a blowing or swelling, known in this country as ,,heft'', ,,rijzing'', ,,pekel-heft'' or ,,vroeg-los'', occurs in the cheese under the press, or during the early stage of brining. As the lactose is converted into lactic acid at a quick rate this production of gas can be only caused by micro-organisms which can develop rapidly. Generally only coli-aerogenes bacteria play a part although then and when it has been noted that also other organisms (yeast and hetero-fermentative lactic acid bacteria) may cause this blowing.

As during this phenomenon the cheese mass has not yet ripened and therefore does not form a homogeneous plastic mass, the occurring holes are irregular in shape. Sometimes a multitude of small holes may arise, unfavourably affecting the aspect of the cheese when cut. Also for this reason the blowing is undesirable. The unsavoury taste produced by the coli-aerogenes bacteria is also a reason for deeming this blowing a fault. All types of the coli-aerogenes group are mostly considered as causal agents of blowing, as nearly all of them are able to ferment lactose with the production of acid and gas. According to WILSON (17) 99% of the coli types, 98% of the intermediate types, and 78% of the cloacae types can ferment lactose with production of acid and gas. Some investigators, however, have reported that not all of the representatives of this group can induce this fault. DORNER (5) reports that the aerogenes bacteria preponderate in the production of gas. WHITEHEAD (16) investigated the action of coli-aerogenes bacteria in Cheddar cheese and he noted that not one of these was able to cause the early gas defect. The results obtained by HÜTTIG (8) in an investigation of soft cheese are, however, quite contradictory to the above. The aerogenes bacteria caused in a high degree the early gas defect, the true coli bacteria not at all. In between were the paracoli bacteria, indicating as such the coli bacteria which lack some characteristic properties of the true coli bacteria such as formation of indole or coagulation of milk.

It seemed to us important to study the behaviour of the various types of the coli-aerogenes group in cheese prepared according to the method practiced in the making of Netherlands kinds of hard cheese and to investigate, why some representatives of this group, although able to produce gas from lactose, do not produce any in cheese. Moreover we have studied the action of some means of prevention in order to settle which would serve this purpose best.

This investigation has been carried out with help of the Foundation ,,Fonds Landbouw Export Bureau 1916—1918" (the L.E.B. fonds) and has been published in detail elsewhere (6). A survey of the principal results is offered here[1]).

From cheese, milk and substances which in dairy practice may come in touch with milk (soil, faeces, grass, fallen leaves) we have isolated 42 strains, which all of them were able to ferment lactose with the production of acid and gas. Table 1 shows the amounts of gas which the different strains could produce from 120 ml lactose broth. After inoculation with some hours' old broth culture of the strain under examination the fermentation bottles were incubated at 30° C. The gas was collected in burettes filled with saturated brine. It appears that the various strains vary but slightly in the amount of gas produced.

In the classification of these strains we followed WILSON (17) who divided the group in three parts by means of the methyl red test, the Voges-Proskauer test and the citrate test. By means of the indole test, the Eijkman test and the liquefaction of gelatine these three main groups are again subdivided. In Table 2 this is shown. In the following we shall often make use of the abbrevations indicated here. Among the strains isolated by us 22 belong to the coli type, 14 to the intermediate and 6 to the aerogenes-cloacae type.

In milk these three main types are of nearly as frequent occurrence. The coli types originate in milk chiefly from contamination with cow dung, as, according to WILSON, 98.4% of the coli-aerogenes bacteria in the latter belong to the coli type. The other types are mostly introduced in the milk by its coming in touch with straw, hay, grass, grains, meal, feeding cakes, etc.

---

[1]) I am greatly endebted to Prof. Dr J. SMIT and Dr Ir K. T. WIERINGA for the interest taken in this investigation and the good advice given.

Table 1.

Production of gas from lactose by the strains isolated.

Strain	ml gas after 24 hours	ml gas after 48 hours	Strain	ml gas after 24 hours	ml gas after 48 hours
1	21.7	28.5	22	32.0	37.6
2	24.3	30.4	23	21.9	28.7
3	36.2	40.4	24	20.2	29.2
4	37.8	47.5	25	22.5	33.0
5	41.9	51.6	26	27.3	33.7
6	32.4	37.0	27	25.8	33.4
7	19.6	30.8	28	8.1	23.9
8	18.8	25.1	29	27.5	35.0
9	29.4	35.7	30	18.1	28.7
10	1.6	22.3	31	27.6	31.5
11	41.4	91.2	32	15.6	33.0
12	20.8	23.0	33	19.4	25.5
13	22.5	28.0	34	6.6	18.4
14	3.9	24.7	35	7.8	22.5
15	20.0	22.9	36	21.9	29.3
16	35.1	41.8	37	28.2	37.8
17	32.2	37.0	38	8.0	12.0
18	22.0	27.6	39	18.9	40.5
19	22.8	48.4	40	5.6	15.5
20	30.0	37.6	41	19.2	24.6
21	33.6	42.6	42	20.2	25.0

Table 2.

Classification of the coli-aerogenes bacteria according to WILSON.

Type	Abbreviations	Indole	M.R.	V.P.	Citrate	Eijkman	Liquefaction of gelatine
B. coli I	C1	+	+	—	—	+	—
B. coli II	C2	—	+	—	—	—	—
Coli-like type 1. . . .	Ca1	+	+	—	—	—	—
Coli-like type 2. . . .	Ca2	—	+	—	—	+	—
Coli-like type 3. . . .	Ca3	—	+	—	—	—	+
Intermediate type I. .	I1	—	+	—	+	—	—
Intermediate type II .	I2	+	+	—	+	—	—
Intermediate-like type	Ia	—	+	—	+	—	+
B. aerogenes I	A1	—	—	+	+	—	—
B. aerogenes II	A2	+	—	+	+	—	—
B. cloacae	C1	—	—	+	+	—	+
Aerogenes-like type 1 .	Aa1	—	—	+	—	—	—
Aerogenes-like type 2 .	Aa2	—	—	+	+	+	—

In a preliminarily test we have endeavoured to settle by means of a combined fermentation and curdling test whether all coli-aerogenes bacteria could cause the early gas defect. In this test bacteria live under practically the same conditions as in cheese. Wide sterile tubes holding about 125 ml, were provided with 1 ml of a three hours' old broth culture of each strain and of 50 ml boiled milk, into which after cooling down to 10° C. we added 1 ml 35% solution of $CaCl_2$ and 0.3 ml rennet (1 : 12.000) per l. The tubes were incubated at 30° C. and curdling was induced in half an hour. After 24 hours we read the result, which did not change by any further incubation. It appeared that under these conditions not all of the strains were able to produce gas and that a characteristic difference in aspect existed between tubes which had and those which had not produced gas. As without any production of gas the curdling action apparently was not strong

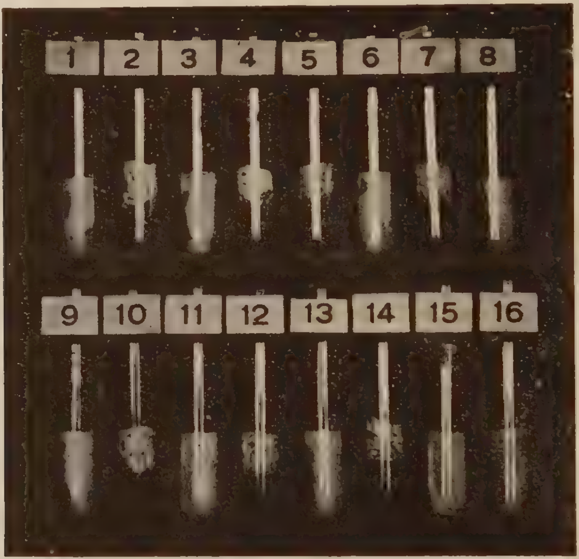


Fig. 1. Curd tests with pure cultures.

Number	Inoculated with	Type	Gas production
1	strain 17	C1	—
2	,, 19	A1	+
3	,, 14	C2	—
4	,, 20	I1	+
5	,, 21	I1	+
6	,, 22	C1	—
7	,, 3	I2	+
8	,, 24	Ca1	—
9	,, 25	C1	—
10	,, 4	I1	+
11	not inoculated		
12	strain 11	A1	+
13	,, 12	C1	—
14	,, 6	I2	+
15	,, 1	C1	—
16	,, 16	C2	—

enough to make the curd contract, tubes in which no or hardly any gas had been formed were filled evenly with curdled milk. In the tubes with gas the curd as a result of the combined action of the rennet and gas had strongly contracted and floated as a spongy mass on top of the whey. Fig. 1 shows some positive and negative fermentation and curdling tests and fig. 2 a section of the curd in a negative and in a positive test. Under these conditions coli types with the exception of one strain (31) could produce no or hardly any gas. The C2 types differed from the C1 types in that they made a few holes in the curd. Among the 14 intermediate strains examined 10 could produce gas in this test, 4 could not. The 6 aerogenes and cloacae strains examined all strongly induced gas production.

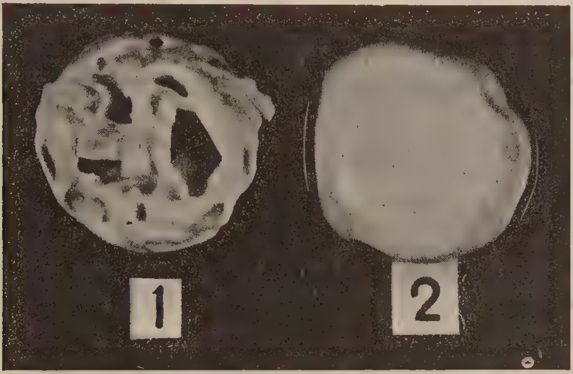


Fig. 2. Section of curds of a positive curd test and a negative one.
Nr. 1. Inoculated with strain 20 (I1).
Nr. 2. Inoculated with strain 22 (C1).

As, however, the conditions under which the bacteria produced gas in the combined fermentation and curdling test differ from those existing in cheese, we may not conclude that the results of this experiment will hold for the early gas defect in cheese. In order to settle this point we have inoculated small lots of pasteurized milk (2.5 l) with the different strains of bacteria and have prepared from each lot a small cheese, following the method of practical cheese making. 5 ml starter was applied to 2.5 l milk, which was further inoculated with 5 ml of a sixteen hours' old milk culture of the strain examined. Outward conditions were chosen such as would favour the occurrence of the early gas defect. Some of the results are shown in Table 3.

The cheeses were judged after 24 hours. In order to ascertain whether the cheese had the defect, it was noted whether it has swelled and a section was examined. A number was fixed according to the following scale:

The cheese has not swelled.	0 = a few holes at most. 1 = fairly many holes. 2 = many holes.
The cheese has swelled and increasingly so in the direction of the arrow.	3 = many holes. 4 = do. 5 = do. ↓

Table 3.

The influence of coli-aerogenes bacteria on the production of gas in cheese.

Cheese	Strain and type	Number of coli-aerogenes bacteria at the curdling	Number of coli-aerogenes bacteria in 24 hours' old cheese	Number of scale	Results fermentation and curdling test	pH
14	1 (C1)	19.000.000/ml	1.600.000.000/g	1	—	5.15
59	27 (C1)	1.900.000 ,,	6.000.000.000 ,,	0	—	5.15
60	30 (C1)	8.000.000 ,,	6.000.000.000 ,,	0	—	5.15
44	26 (C2)	1.300.000 ,,	600.000.000 ,,	0	—	5.20
79	2 (C1)	1.900.000 ,,	—	1–2	—	5.20
54	31 (C1)	800.000 ,,	—	3	+	5.35
9	11 (A1)	600.000 ,,	190.000.000 ,,	4	+	
10	39 (C1)	2.500.000 ,,	80.000.000 ,,	4	+	
23	5 (I1)	400.000 ,,	—	4	+	
2	21 (I1)	—	—	4	+	4.90

The cheeses 44, 79 and 23 are shown in fig. 3, 4 and 5 (No. 1 = cheese 44, No. 33 = cheese 79 and No. 19 = cheese 23). Moreover the fig. 8, 9 and 10 show some specimens with early gas defect.

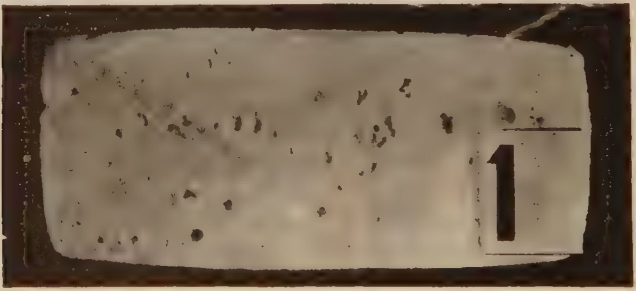


Fig. 3. Milk inoculated with 5 ml culture of strain 26 (C2).

Here again we note a great difference in the measure in which different strains induce blowing. The strains which give negative results in the fermentation and curdling test were neither able to form much gas in cheese. So we can classify the bacteria of the coli-aerogenes group in blowing-positive and blowing-negative types, the former inducing no or hardly any blowing and the latter strong blowing. In our experiments the coli types mostly belonged to the blowing-negative type and the aerogenes and cloacae strains to the blowing-positive type. The intermediate strains were one or the other. In the fermentation and curdling test we have a simple method for predicting whether a definite bacterium from the coli-aerogenes group will cause blowing.

Fig. 4. Milk inoculated with 5 ml culture of strain 2 (C1).

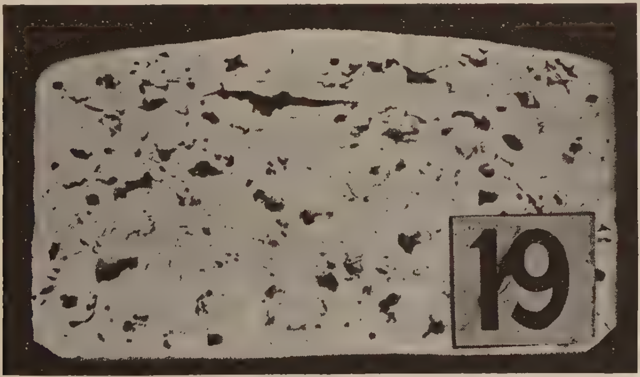


Fig. 5. Milk inoculated with 5 ml culture of strain 5 (I1).

All of the strains, however, caused an unsavoury taste and thus the occurrence of coli-aerogenes bacteria is always undesirable, even without their inducing any blowing.

Therefore in a general way no correlation will exist between the number of coli-aerogenes bacteria in milk and the measure in which early blowing occurs in the cheese made therefrom. Neither will any correlation exist between early gas defect and the number of coli-aerogenes bacteria in cheese. This has been reported also by YALE and MARQUARDT (19), although they offer another cause, which in our opinion is not the sovereign one.

We could further note a great difference in the degree in which various types will remain alive in cheese. Bacteria of the C1 type survive over a long period, those of the C2 type as well as those of the intermediate and the aerogenes-cloacae types die off rapidly. HÜTTIG (8) and CROSSLEY (4) report the like.

Now the question arises how these results may be explained, as according to the data taken up in Table 1, all strains were able to produce gas from lactose broth. Moreover according to Table 3 bacteria of the blowing-negative type do not develop in cheese in a lesser degree than bacteria of the blowing-positive type. This holds as well for the fermentation and curdling test. In order to settle this point we estimated first of all the amount of gas our strains could produce in whey, as production of gas therein can more adequately be compared with gas produced in cheese than in lactose broth. The results are recorded in Table 4 in which for the sake of comparison the results of the fermentation and curdling test have also been inserted.

We see that the results of these tests, which could always be reproduced, agree well with those of the fermentation and curdling test. The strain which in the latter experiment had furnished positive results, also produced much gas from whey although it is true that in the production from whey the differences do not show sharply before the lapse of 24 hours (*cf.* the production of gas by the strains 5, 9, 20 and 39) whilst the result of a fermentation and curdling test was already definite after 24 hours. It shows clearly that in whey, cheese and the fermentation and curdling test the production of gas by the blowing-negative bacteria is inhibited. So we have surmised that this might be owing to substances occurring in the milk.

Considering the composition of milk it occurred to us that it might be citric acid, which acted as such. In fact milk contains 0.14—0.20% of this acid. In order to settle this point we made up a liquid which in composition as well as in properties corresponded as much as possible to skim milk. We used the method of PORCHER (11). By means of stirring we solved 30 g casein, prepared according to HAMMERSTEN and 1100 mg CaO in 1 l water. The pH then exceeded 7 as under these conditions about 600 mg CaO is needed to obtain a neutral solution of casein. Subsequently we added 0.2 N H_3PO_4 in drops until the pH had dropped to 7. Owing to the casein-calcium-phosphoric acid complex formed the liquid became white and opaque. In order to arrive at a still closer correspondence to skim milk we added 0.45% albumine and the salts occurring in milk. To part of this synthetic skim milk we added 0.16% citric acid as sodium citrate. The final pH was 6.8. With this skim milk we carried out a fermentation and curdling test such as it has been described above. Part of the

Table 4.

Production of gas from 40 ml whey by the strains isolated.

Strain	Type	ml gas formed after			Result of the fermentation and curdling test
		24 hours	48 hours	72 hours	
1	C1	1.0	7.0	7.2	—
2	C1	2.0	5.5	6.1	—
3	I2	17.5.	29.0	31.9	+
4	I1	17.5	70.0	110.0	+
5	I1	2.5	13.5	22.6	+
6	I2	14.5	24.5	28.1	+
7	C1	1.5	3.0	4.3	—
8	C1	1.5	4.0	4.6	—
9	C1	1.5	7.5	8.6	—
10	C1	0.5	0.6	0.6	—
11	A1	10.0	55.0	92.0	+
12	C1	0.3	2.5	3.6	—
13	C1	0.3	1.5	2.0	—
14	C2	0.3	1.3	2.9	—
15	I1	1.0	3.5	6.5	—
16	C2	1.4	6.4	8.0	—
17	C1	1.0	3.7	3.5	—
18	C1	5.9	7.6	7.6	—
19	A1	2.0	25.0	51.5	+
20	I1	5.4	57.8	121.0	+
21	I1	7.7	25.5	45.5	+
22	C1	1.4	3.1	4.2	—
23	Ca1	0.5	2.5	3.8	—
24	Ca1	0.7	1.6	1.9	—
25	C1	2.2	4.0	5.2	—
26	C2	1.2	4.5	6.8	—
27	C1	1.5	4.0	4.5	—
28	I2	10.0	19.3	35.0	+
29	C1	3.3	5.3	6.9	—
30	C1	0.6	1.0	2.4	—
31	C1	7.3	9.4	10.6	±
32	I2	6.0	9.7	12.6	+
33	I1	1.0	3.6	4.6	±
34	I1	0.8	1.0	1.4	—
35	I1	0.3	1.0	1.5	—
36	C1	2.6	2.7	2.8	—
37	I2	15.6	26.1	39.5	+
38	I1	0.4	0.8	1.0	—
39	C1	2.8	35.6	61.6	+
40	Aa1	7.0	10.1	15.5	+
41	Aa1	8.6	22.2	40.1	+
42	Aa1	8.2	17.3	32.1	+

results are recorded in fig. 6. In the absence of gas here again the tube contained evenly clotted milk, whilst when gas did occur a spongy mass of curd floated on top of the liquid. The openings, however, were much smaller, but much more numerous than in a similar test with cow's milk. Without citric acid all strains produced gas. When citric acid had been supplied strains which could not form gas in whey or cheese neither did so here. Thus

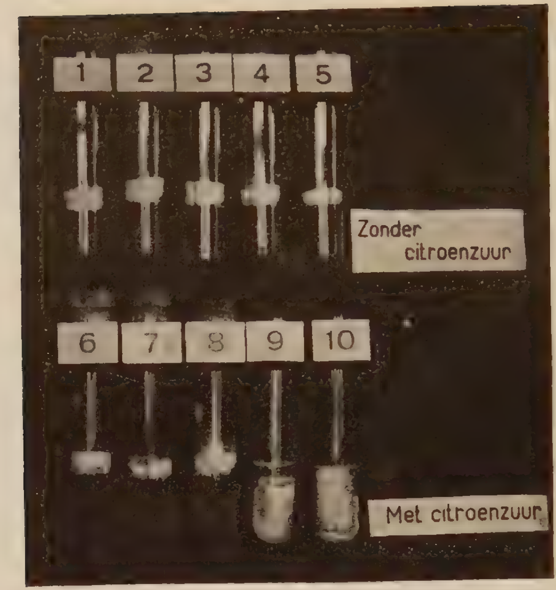


Fig. 6. Curd tests with synthetic skim milk alternately containing citric acid.

Number	Inoculated with	Containing	Gas production
1	strain 11 (A1)	no citric acid	+
6	,,	0.16% ,,	+
2	strain 20 (I1)	no ,,	+
7	,,	0.16% ,,	+
3	strain 37 (I2)	no ,,	+
8	,,	0.16% ,,	+
4	strain 2 (C1)	no ,,	+
9	,,	0.16% ,,	—
5	strain 24 (Ca1)	no ,,	+
10	,,	0.16% ,,	—

it seemed probable that the deviating behaviour of blowing-negative types would be owing to the content of citric acid in milk. We will, however, not exclude the possibility that other substances acting in a similar way might occur in milk.

In order to study the mode of action of citric acid more closely we have estimated the gas produced by the different types from lactose broth or lactose-yeast extract unto which increasing volumes of a neutral sterile 10% solution of citric acid had been added. Some of the results obtained are recorded in Tables 5 and 6.

The production of gas by blowing-negative strains was delayed somewhat by the presence of citric acid. As to the other strains the citric acid initially did not act on their production of gas. Some difference in the amounts for instance after 18 hours did occur, but these were accidental and occurred as well in the absence of citric acid. Of greatest importance is the fact that the onset of gas production by the blowing-positive strain was not delayed

Table 5.

Production of gas by strain 15 (I1), a blowing-negative strain from lactose broth unto which citric acid had been added.

Citric acid added	ml gas produced after			
	18 hours	24 hours	42 hours	48 hours
—	10.0	12.0	13.5	13.5
0.05%	7.9	9.7	11.3	11.4
0.10%	4.0	6.1	8.6	8.9
0.20%	0.5	1.2	2.5	2.7

Table 6.

Production of gas by strain 11 (A1), a blowing-positive strain from lactose broth unto which citric acid had been added.

Citric acid added	ml gas produced after			
	18 hours	24 hours	42 hours	48 hours
—	8.6	11.4	16.4	17.6
0.05%	11.4	16.7	33.0	38.1
0.10%	7.6	12.4	27.9	32.4
0.20%	9.1	17.0	40.6	48.2
0.50%	4.8	14.4	42.7	50.9

by an addition of citric acid. Later on, however, for these types the amount of gas increased with increasing amounts of citric acid added and so the final production of gas in broth containing citric acid by blowing-positive strains was much larger than the production by other strains.

In another experimental set next to the production of gas we have estimated the decrease in the amount of citric acid. The method of PUCHER, SHERMAN and VICKERY (12) has been followed which allows the estimation of 0.1—1.0 mg. In cultures of blowing-negative bacteria no substances occurred disturbing this estimation. In an experiment with strain 2 (C1) we used 3 fermentation bottles of 550 ml which were provided with 4 ml of a few hours' old broth culture and further filled with lactose yeast extract. Bottle II was supplied with 0.05% and bottle III with 0.20% citric acid as sodium citrate. In Table 7 the results are recorded.

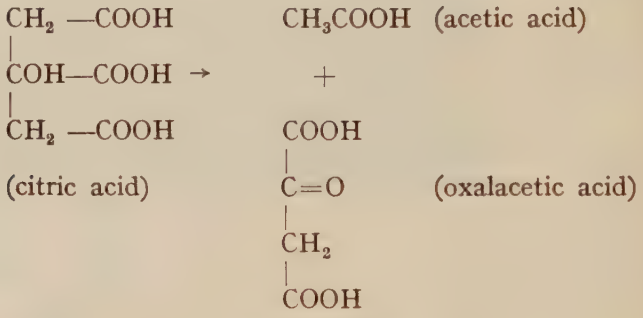
So the blowing-negative bacteria could not start with the production of gas from lactose before the citric acid had been used up. It seems to us that this might be explained by assuming that citric acid acts as a hydrogen acceptor, the fermentation of sugar thus taking another course and producing hardly any gas. Citric acid might accept hydrogen in the following way.

Table 7.

The influence of citric acid on the production of gas by the blowing-negative strain 2 (C1) from lactose yeast extract.

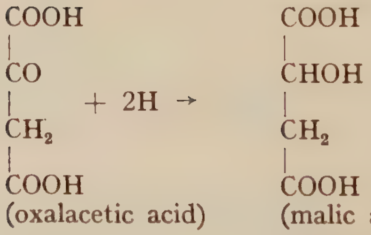
Fermentation bottle	Culture solution	ml gas after					% citric acid	
		16 h.	19 h.	21 h.	24 h.	41 h.	0 h.	16 h.
I	Lactose yeast extract	26.0	35.2	41.0	49.0	75.0	—	—
II	Idem + 0.05% citric acid	4.0	11.2	13.2	18.5	38.5	0.05	0.00
III	Idem + 0.20% citric acid	3.0	5.0	5.7	11.5	23.0	0.20	0.00

I.



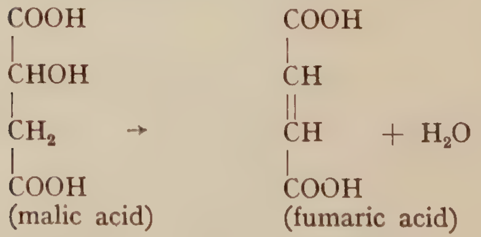
$$\begin{array}{l} CH_2-COOH \\ | \\ COH-COOH \\ | \\ CH_2-COOH \\ \text{(citric acid)} \end{array} \rightarrow \begin{array}{ll} CH_3COOH & \text{(acetic acid)} \\ + & \\ COOH & \\ | & \\ C=O & \text{(oxalacetic acid)} \\ | & \\ CH_2 & \\ | & \\ COOH & \end{array}$$

II.



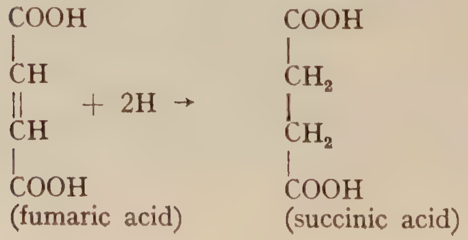
$$\begin{array}{l} COOH \\ | \\ CO \\ | \\ CH_2 \\ | \\ COOH \\ \text{(oxalacetic acid)} \end{array} + 2H \rightarrow \begin{array}{l} COOH \\ | \\ CHOH \\ | \\ CH_2 \\ | \\ COOH \\ \text{(malic acid)} \end{array}$$

III.



$$\begin{array}{l} COOH \\ | \\ CHOH \\ | \\ CH_2 \\ | \\ COOH \\ \text{(malic acid)} \end{array} \rightarrow \begin{array}{l} COOH \\ | \\ CH \\ \| \\ CH \\ | \\ COOH \\ \text{(fumaric acid)} \end{array} + H_2O$$

IV.



$$\begin{array}{l} COOH \\ | \\ CH \\ \| \\ CH \\ | \\ COOH \\ \text{(fumaric acid)} \end{array} + 2H \rightarrow \begin{array}{l} COOH \\ | \\ CH_2 \\ | \\ CH_2 \\ | \\ COOH \\ \text{(succinic acid)} \end{array}$$

This assumption may be substantiated by a number of arguments. Reaction I is not unusual for coli-aerogenes bacteria, for Brewer and Werkman (3) consider this as the initial reaction in the dissimilation of citric acid by *Aerobacter indologenes.* Krebs (9) reports that coli bacteria, inter alia by means of the enzyme fumarase may reduce oxalacetic acid into fumaric acid. The same author reports that coli bacteria by means of the enzyme succinodehydrogenase may further reduce fumaric acid into succinic acid.

If the above assumption is right the intermediate products, oxalacetic acid, malic acid and fumaric acid must act in the same way as does citric acid. Then it must be possible by means of these compounds to delay the onset of gas production from lactose up till the moment these acids will have disappeared. In fact we were able to realize this. Fermentation bottles holding 120 ml were provided with 1 ml of a some hours' old broth culture of the strain to be tested. To part of the bottles we further added 1½ ml of a neutralized sterile solution of either acid. The bottles were further filled with lactose yeast extract and incubated at 30° C. Tables 8 and 9 offer some results.

Table 8.

The influence of malic acid on the production of gas from lactose yeast extract by blowing-negative strains.

Strain	Added	ml gas produced after					
		12½ h.	14½ h.	17 h.	21 h.	37½ h.	61½ h.
2 (C1)	—	7.2	10.0	13.8	18.1	26.5	29.2
2 (C1)	0.12% malic acid . .	0.3	0.8	1.6	4.0	10.5	14.2
22 (C1)	—	3.5	5.5	8.0	10.5	15.5	17.5
22 (C1)	0.12% malic acid . .	0.2	0.9	1.5	2.5	5.0	7.0

Table 9.

The influence of oxalacetic acid and fumaric acid on the production of gas from lactose yeast extract by blowing-negative strains.

Strain	Added	ml gas produced after					
		12 h.	14 h.	17 h.	19 h.	25 h.	37 h.
22 (C1)	—	3.5	5.6	9.1	12.3	17.5	22.0
22 (C1)	0.12% fumaric acid	0.6	1.5	3.0	5.0	8.2	12.5
22 (C1)	0.12% oxalacetic acid	0.5	0.5	1.5	2.8	5.7	10.0
7 (C1)	—	2.5	5.0	8.5	12.0	17.5	25.0
7 (C1)	0.12% fumaric acid	0.3	0.7	1.5	2.5	4.8	8.2

Finally we tried to settle whether by means of the supply of citric acid a larger production of succinic acid was induced. Each molecule of citric acid should furnish one molecule of succinic acid. We inoculated 6 fermentation bottles filled with lactose yeast extract with the blowing-negative strains 2, 22 and 7. To half of the bottles we added 0.15% citric acid as sodium citrate. Directly after the disappearance of the citric acid we estimated the content of succinic acid of these culture solutions. Per 100 ml 150 mg citric acid had been present initially and thus in the solution provided with citric acid 92 mg succinic acid should have been formed over and above the amount present in the other cultures, assuming that a same amount of succinic acid was formed from lactose and yeast extract in either case. Table 10 offers the results. We followed the method described by Smit (15).

Table 10.

Increase in yield of succinic acid following the addition of citric acid to cultures on lactose yeast extract of blowing-negative strains.

Strain	mg succinic acid per 100 ml		Increase in yield by the addition of citric acid
	lactose yeast extract	lactose yeast extract with 15% citric acid	
2 (C1)	98.2	219.8	116.6 mg
22 (C1)	99.2	190.1	90.9 mg
7 (C1)	96.2	186.9	90.7 mg

Here again the close agreement pleads for the assumption made. Still some questions are left unanswered. The data in Table 7 show that under these conditions 0.20 % citric acid acted but slightly more strongly than did 0.05%. This, however, cannot be explained by the scheme we made up. Moreover it appeared that in lactose yeast extract cultures of blowing-negative strains sometimes after the addition of 0.20% citric acid finally more gas had been produced than after the addition of 0.10%. Further data are offered in Table 11.

Table 11.

Production of gas by strain 26 (C2) from cultures on lactose yeast extract, unto which citric acid had been added.

Citric acid added	ml gas produced after						
	16 h.	24 h.	40 h.	48 h.	64 h.	72 h.	96 h.
—	3.0	6.1	8.2	8.6	8.7	9.0	9.0
0.025%	1.0	2.5	4.5	4.6	5.2	5.6	5.8
0.050%	0.6	1.0	1.0	1.3	1.6	1.8	2.0
0.100%	0.4	0.5	1.0	1.2	1.2	1.5	1.8
0.200%	0.4	1.2	1.6	1.5	3.5	4.1	5.3

Neither this fact can be explained by the scheme offered. We shall offer an explaination of this phenomenon after having reported what happens with the citric acid added to cultures of blowing-positive strains. For this we can make use of a paper by Brewer and Werkman (3). These investigators have described the way in which *Aerobacter indologenes*, an aerogenes and thus a blowing-positive type, ferments a mixture of sugar and citric acid. The fermentation has been brought about in a liquid containing per l 100 m.mol. glucose, 50 m.mol. citric acid and 5 g peptone. In Table 12 the results as far as they are of interest to us are recorded.

Table 12.

The fermentation of glucose together with citric acid by *Aerobacter indologenes*.

Time after	Disappeared (in m.moles)		Produced (in m.moles)	
	glucose	citric acid	hydrogen	carbon dioxide
17 h.	41.8	0.83	4.5	58.2
23 h.	55.6	1.54	7.5	78.1
36 h.	78.3	2.73	18.6	120.0
50 h.	100.0	3.46	36.5	166.1
161 h.	100.0	49.4	63.3	252.2
300 h.	100.0	50.0	67.1	266.2

Initially no citric acid disappears, and so an addition of this acid to cultures of blowing-positive strains cannot act on the production of gas, this agreeing with our findings reported in Table 6. This initial non-fermenting of citric acid, in the presence of sugar differs widely from the rapid disappearance of citric acid in the fermentation of sugar by blowing-negative strains. Lateron the citric acid does disappear and therefore the total production of gas increases. We may assume that in our experiments with blowing-positive cultures the bacteria along with the fermentation of sugar proceeded with the fermentation of citric acid, this explaining the increase in the production of gas reported. This does not mean, however, that both fermentations need to have taken an independent course, cross actions may actually have occurred. Now we can also explain the difficulties we encountered in the investigation of the influence of citric acid on the fermentation of lactose by blowing-negative bacteria. We told before that in some cultures 0.05% citric acid acted about as strongly on the gas production as 0.20% and that in lactose yeast extract the gas production following the addition of 0.20% citric acid might exceed that value after a supply of 0.10 %. It might seem as if this could not be accounted for by means of the scheme we offered for the action of citric acid. It appears plausible, however, to assume that after a large supply of citric acid not all of the acid will have been used up as hydrogen acceptor but after some lapse of time part of the citric acid will have been fermented by the blowing-negative bacteria, in this respect behaving similarly to blowing-positive bacteria. Assuming this the above mentioned deviation can be easily explained. This explaination is further substantiated by the fact that blowing-negative bacteria, when taken from a usual nutrient medium will not develop in the citrate solution according to KOSER. They will, however, be able to do so, when bacteria are used, which have developed in a solution containing lactose and citric acid (for instance whey). So in nutrient solution with lactose when provided with citric acid, coli bacteria will finally ferment part of the citric acid, thus causing an increase in the production of gas. The amount of citric acid used as hydrogen acceptor will probably be closely connected with the amount of sugar that can be fermented in the culture solution. When this amount is larger, more citric acid will serve as hydrogen acceptor and the bacteria will proceed to the fermentation of the acid later. In this connection we may assume that in the fermentation and curdling test and in cheese in which owing to the buffering action of the medium the bacteria can ferment more sugar, generally a larger part if not all of the available acid will be used as hydrogen acceptor, thus preventing the occurrence of gas.

Finally we have tried to show that citric acid in cheese acts in the same way as in liquid cultures. We were not able, however, to estimate the citric acid in cheese, as substances occur herein which disturb the usual analytical methods for its estimation. We could, however, show that after inoculation with blowing-negative bacteria the citric acid was about just sufficient to inhibit the occurrence of the early gas defect. After inoculation with a blowing-negative strain we were able, by means of a systematic drawing off of the whey during the cheese making and a supply of a $4\frac{1}{2}\%$ solution of lactose, to decrease the concentration of citric acid in the curd in such measure that the early gas defect made its appearance.

Moreover in cheeses which had been inoculated with blowing-negative bacteria we could note sometimes a weak form of the early gas defect when during the preparation much water had been employed. If, however, instead of water a 0.18% solution of citric acid had been used the cheese was practically free from holes.

The latter experiments make plausible that actually in cheese the citric acid acts on the fermentation of sugar by the blowing-negative bacteria in the same way as in the experiments with liquid cultures described above.

In our experiments with cheese we have mostly used a starter containing only bacteria of the species *Streptococcus lactis*. In practical cheese making starters are often in use which also contain the aroma bacteria decomposing citric acid. It might be thought that the aroma bacteria by their disposing of the citric acid would offer the blowing-negative bacteria the possibility to induce the early gas defect. Several times we have made cheese by means of a starter which next to the usual lactic acid streptococci contained aroma bacteria; no influence, however, of the latter could be noted. The aroma bacteria decompose citric acid most strongly in an acid medium and under those conditions the action of the coli-aerogenes will as a rule have been brought to a close. The citric acid has already been disposed of by the blowing-negative bacteria, when the aroma bacteria have developed sufficiently to proceed with its decomposition.

In all experiments described thus far we have always used as inoculum either blowing-negative or blowing-positive bacteria and thus the question is still left open what will happen when the milk has been contaminated with both types of bacteria as will often be the case in practical cheese making.

In order to answer this question we have first of all run some preliminary fermentation and curdling tests. In each experimental set we used 4 culture tubes which were provided with 0.001 ml of a broth culture of a blowing-negative strain. To one tube we did not add any further bacteria, to the second 0.01 ml of a broth culture of a blowing-positive strain, to the third 0.1 ml and to the fourth 1 ml of the same strain was given. So we had realized the ratios 1 : 0, 1 : 10, 1 : 100 and 1 : 1000.

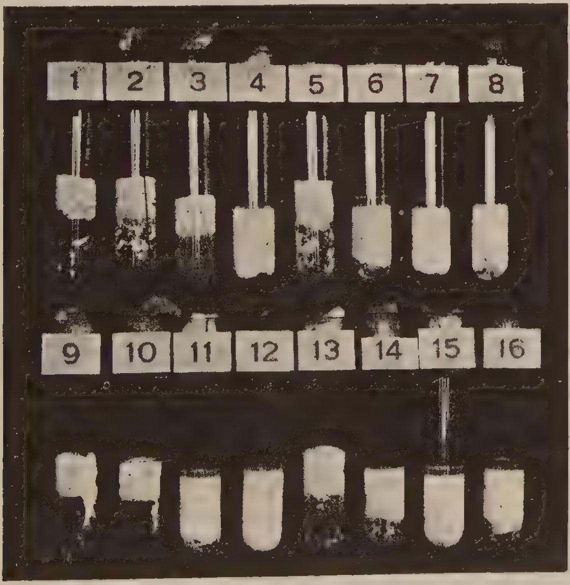


Fig. 7. Curd tests with mixtures of blowing-positive and blowing-negative ones. Table 13 shows further indications.

For estimation of the gas production from whey we followed the same method. In fig. 7 and Table 13 the results of some experiments are recorded.

Table 13.
Influence of combinations of blowing-positive and blowing-negative bacteria.

Combination of		Ratio	Fermentation and curdling test	No. on fig. 7	ml gas produced from 40 ml whey after:		
blowing-positive strain	blowing-negative strain				24 h.	48 h.	72 h.
11 (A1)	2 (C1)	1 : 0	+	1	4.2	15.9	25.0
id.	id.	1 : 10	+	2	2.0	13.7	48.4
id.	id.	1 : 100	±	3	2.3	5.5	11.4
id.	id.	1 : 1000	—	4	0.7	1.9	2.5
20 (I1)	2 (C1)	1 : 0	+	5	5.2	21.4	44.0
id.	id.	1 : 10	±	6	1.2	2.4	3.0
id.	id.	1 : 100	—	7	1.5	3.2	4.1
id.	id.	1 : 1000	—	8	1.5	2.6	3.0
37 (I2)	2 (C1)	1 : 0	+	9	6.9	15.2	21.2
id.	id.	1 : 10	+	10	0.7	2.0	3.3
id.	id.	1 : 100	—	11	0.5	1.7	2.5
id.	id.	1 : 1000	—	12	1.2	2.2	3.0
20 (I1)	15 (I1)	1 : 0	+	13	5.7	14.9	24.6
id.	id.	1 : 10	±	14	2.5	5.5	6.8
id.	id.	1 : 100	—	15	1.1	2.5	3.1
id.	id.	1 : 1000	—	16	3.1	6.2	6.8

These experiments show that a blowing-positive culture cannot produce any gas when a certain number of blowing-negative bacteria are present as well. The various combinations differ mutually as may be read from the results. Thus the action of strain 11 (A1) could hardly be weakened by the presence of a blowing-negative culture whilst strain 20 (I1) was strongly influenced.

We have also investigated whether this phenomenon would hold as well for the early gas defect in cheese. Two lots of milk were inoculated with a milk culture of a blowing-positive strain. One lot moreover received a culture of a blowing-negative type. From either lot of milk cheese was made in the same way, the cheese being judged after 24 hours. Table 14 offers some results.

Although marked differences appeared to exist between the various combinations, we may state that generally the presence of blowing-negative bacteria will strongly lessen the occurrence of the early gas defect. The blowing positive-strains during the initial period do not act upon the citric acid, so that the blowing-negative bacteria may dispose of it freely and convert the sugar into acid without any appreciable production of gas.

Table 14.

On the influence of combinations of blowing-positive and blowing-negative strains on the production of gas in cheese.

Cheese	Added to a culture of a		Judgment	pH
	blowing-positive strain	blowing-negative strain		
44	$\frac{1}{4}$ ml strain 11 (A1)	$\frac{10}{4}$ ml strain 2 (C1)	3—4	5.20
43	do.	—	3—4	5.20
61	$\frac{1}{2}$ ml strain 39 (C1)	—	4	5.10
62	do.	$\frac{5}{2}$ ml strain 26 (C2)	2	5.10
65	$\frac{1}{2}$ ml strain 19 (A1)	—	4	5.15
66	do.	$\frac{5}{2}$ ml strain 30 (C1)	0	5.15

The cheeses 65 and 66 are shown in fig. 8 (cheese 65 = No. 31 and cheese 66 = No. 32).

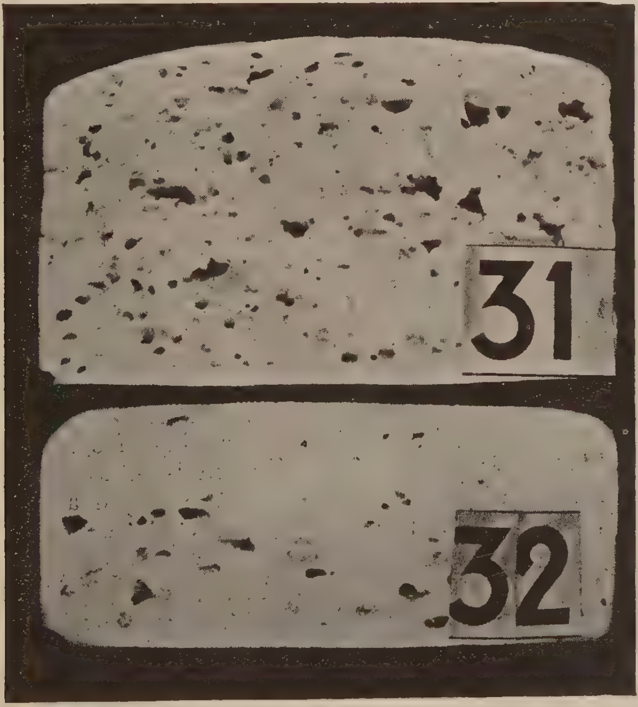


Fig. 8. The influence of mixtures of blowing-positive strains and blowing-negative ones on the gas production in cheese. Nr. 31. Milk inoculated with 0.5 ml culture of the blowing-positive strain 19 (A1). Nr. 32. Milk inoculated with 0.5 ml culture of the same strain and moreover supplied with 2.5 ml culture of the blowing-negative strain 30 (C1).

In order to explain the influence of the blowing-negative bacteria on the production of gas of the blowing-positive types we might consider two causes:

1. The blowing-negative bacteria producing more acid might affect the blowing-positive by means of this acid. In this respect the blowing-negative bacteria in cheese might function as precursors of the lactic bacteria, which as a rule bring the activity of the gas producing bacteria to a close.

2. One or more metabolic products of the blowing-negative bacteria might be able to alter the fermentation of sugar by the blowing-positive bacteria in a way that less gas is formed. Here we think of acetic acid which is a fairly important metabolic product of the blowing-negative bacteria. REYNOLDS, JACOBSSON and WERKMAN (14) found that acetic acid, when added to a fermentation of glucose by *Aerobacter indologenes*, was reduced and thus no further hydrogen could be produced from sugar by this bacterium. The reduced acetate was entirely recovered as 2—3 butylene glycol. MICKELSON and WERKMAN (10) could further show that this reduction of acetic acid merely occurs in media with a pH below 6.3. As in the fermentation and curdling tests and in the production of gas in cheese we meet with an acid medium it is very probable that here as well this reduction of acetate plays an important part in the suppression of the gas production, the more so as hydrogen gas owing to its slight solubility plays a very important part in the fermentation and curdling tests and in cheese.

After having studied the course of the early gas defect we have also studied the means of combat of this fault. Of course best of all is winning of the milk as cleanly as possible, thus preventing the introduction of blowing-positive bacteria. These bacteria can further be killed off by pasteurization but this treatment has its disadvantages, whilst often the means for pasteurization will not be available. The milk once contaminated with blowing-positive bacteria, conditions during the making of cheese may be regulated in a way that their development is inhibited as much as possible. We mention in this recpect the use of an increased amount of starter and a rapid brining of the cheese. Still it is not always possible by these means to prevent entirely the occurrence of the early gas defect. In practical cheese making potassium nitrate has been applied since long, this substance generally being added to the milk during curdling. Various investigators could show the favourable action of nitrate (2, 7, 8). HÜTTIG (8) reported that $KClO_3$ as well was a suitable means of prevention of the defect, whilst WOJTKIEWICZ (18) proposed the use of nitrite.

So we have studied the action of these substances in order to ascertain which would serve best. Moreover we have studied whether $KBrO_3$ and $K_2S_2O_8$ might be suitable. This, however, appeared not to be the case.

It appeared that nitrate was rapidly reduced to nitrite by coli-aerogenes bacteria and that subsequently the nitrite as well disappeared, it being further reduced and its nitrogen either assimilated or converted into ammonia. As long as the bacteria fermented sugar, nitrogen was assimilated; thereupon the formation of ammonia sets in. It appeared further that the bacteria in the initial period, namely as long as they fermented sugar, were not impaired by these reductions. During the formation of ammonia, however, they died off. In order to study the influence of ammonia on the gas production we used fermentation bottles of 40 ml. After having provided these with 0.05 ml of a some hours' old broth culture of a certain strain and with a definite volume varying from 0—1 ml of a sterile solution of KNO_3 we filled these with whey. The bottles were incubated at 30° C.; the production of gas was read after various intervals and the presence of nitrite and in its absence of nitrate was reacted upon. Some of the data obtained are recorded in the tables 15 and 16.

Table 15.
Influence of KNO_3 on the production of gas from whey by strain 11 (A1).

Lapse of time	ml gas produced in bottles to which per 100 l had been added:				
	0 g KNO_3	20 g KNO_3	60 g KNO_3	80 g KNO_3	100 g KNO_3
after 13 hours	6.0	1.0	0.2 ——— 1)	0.1	0.1
,, 16 ,,	10.3	2.6	1.5	0.5 ——— 1)	1.0
,, 21 ,,	25.6	7.2	5.6	3.8	2.6 ——— 1)
,, 37 ,,	98.5	40.0	47.5	41.5	12.0
,, 42 ,,	115.0	53.8	63.0	64.5	24.2
,, 48 ,,	130.0	66.6	77.4	86.2	43.6

1) These lines indicate the moment in which the nitrate and the nitrite formed from it has disappeared. In the experiment with 20 g KNO_3 this moment has not been exactly determined as it occurred within 13 hours

Table 16.
Influence of KNO_3 on the number of bacteria of strain 11 (A1) in whey.

KNO_3 added in g per 100 l	Number of bacteria after 20.5 hours	Turbidity after 68 hours
0	50.10^8 per ml	IV 1)
20	41.10^8 ,, ,,	IV
60	38.10^8 ,, ,,	IV
80	31.10^8 ,, ,,	IV
100	52.10^8 ,, ,,	IV

1) The culture without addition of nitrate we indicate as IV. Lower figures indicate a weaker turbidity.

It appears from these data that without any inhibition of the development of the bacteria we could delay the onset of the production of gas over a certain period. In experiments with other blowing-positive bacteria we obtained the same results. The nitrate obviously acts as a hydrogen acceptor and the fermentation of sugar owing to the hydrogen being used in the reduction of nitrate, takes another course and hardly any gas is produced.

WOJTKIEWICZ assumed that nitrate prevents the production of gas by means of the nitrite it gives rise to which would cause the death of the bacteria. Therefore he proposed to apply KNO_2 directly. Our experiments show that he was wrong and that nitrate only becomes toxic when the bacteria no longer dissimilate any sugar and the reduction into NH_3 has set in. In the above experiment with strain 11 this stage was not reached as nitrate and nitrite had already entirely disappeared.

It is to be expected that KNO_2 may as well influence the gas production, as it also acts as a hydrogen acceptor. As the data of Tables 17 and 18 show, nitrite actually could defer the onset of production of gas over a certain period, without any toxic action coming into play. It does appear, however, that whenever after a certain period, *viz.*, when no further sugar is dissimilated and the production of NH_3 has set in, still some nitrite is left, the growth of bacteria is brought to a close and the turbidity of the culture grows less dense.

Table 17.

Influence of KNO_2 on the production of gas from whey by strain 11 (A1).

Lapse of time	ml gas produced in bottles to which per 100 l had been added:					
	0 g KNO_2	20 g KNO_2	40 g KNO_2	60 g KNO_2	80 g KNO_2	100 g KNO_2
after 12 hours	4.9	0.2	0.2	0.1	0.1	0.1
		——— 1)				
„ 15 „	8.0	0.6	0.2	0.1	0.1	0.1
			——— 1)			
„ 20 „	22.3	3.6	0.5	0.2	0.1	0.1
„ 25½ „	52.3	19.4	2.6	0.2	0.2	0.2
				——— 1)		
„ 36½ „	110.0	63.0	17.6	3.6	1.0	0.5
					——— 1)	
„ 45 „	150.0	89.0	34.4	9.5	1.7	0.6
„ 62 „	220.0	138.0	102.0	41.5	8.3	2.3
„ 73 „	263.0	165.0	132.0	63.0	24.5	5.0
						——— 1)
„ 85 „	300.0	181.0	161.0	86.0	51.0	8.8
„ 94 „	327.0	194.0	186.0	108.0	77.0	15.5

1) See note Table 15.

Table 18.
Influence of the addition of KNO_2 to whey on the number of bacteria of strain 11 (A1).

KNO_2 added in g per 100 l	Number of bacteria after 22 hours	Turbidity after 94 hours
0	18.10^8 per ml	IV 1)
20	26.10^8 ,, ,,	IV
40	50.10^8 ,, ,,	IV
60	34.10^8 ,, ,,	IV
80	28.10^8 ,, ,,	IV
100	21.10^8 ,, ,,	III

1) See note Table 16.

So both potassium nitrate and nitrite can serve in the combat of the early gas defect. We have to add these substances in such amounts that the onset of the gas production will be deferred up till the moment in which lactic acid bacteria owing to their production of acid will be able to inhibit the activity of the blowing-positive bacteria. By means of experiments with cheese as well we have ascertained the action of these substances. Per 2.5 l milk we inoculated 1 ml culture of a blowing-positive strain. From either of two lots of milk (2.5 l) we made a cheese, having added to one lot either nitrate or nitrite, the other lot serving as a control. Table 19 offers some results.

Table 19.
Influence of KNO_3 and KNO_2 on the production of gas in cheese.

Cheese	Strain	Added per 100 l	After 24 hours			
			Reaction on KNO_2 and KNO_3	Judgment	Number of blowing-positive bacteria per g	pH
109	11 (A1)	—		4	25.000.000	5.20
110	id.	40 g KNO_3	—	3—4	60.000.000	5.15
115	11 (A1)	—		4	250.000.000	5.00
116	id.	100 g KNO_3	+	0	250.000.000	5.40
141	21 (I1)	—		5	250.000.000	5.20
142	id.	50 g KNO_3	—	3—4	250.000.000	5.20
145	21 (I1)	—		4	60.000.000	5.05
146	id.	80 g KNO_3	+	0	10.000.000	5.55
143	21 (I1)	—		4	60.000.000	5.10
144	id.	50 g KNO_2	—	3	60.000.000	5.10
147	21 (I1)	—		4	60.000.000	5.05
148	id.	80 g KNO_2	+	0	60.000.000	5.55

Fig. 9 shows the cheeses 115 and 116 (No. 41 = cheese 115 and No. 42 = cheese 116).

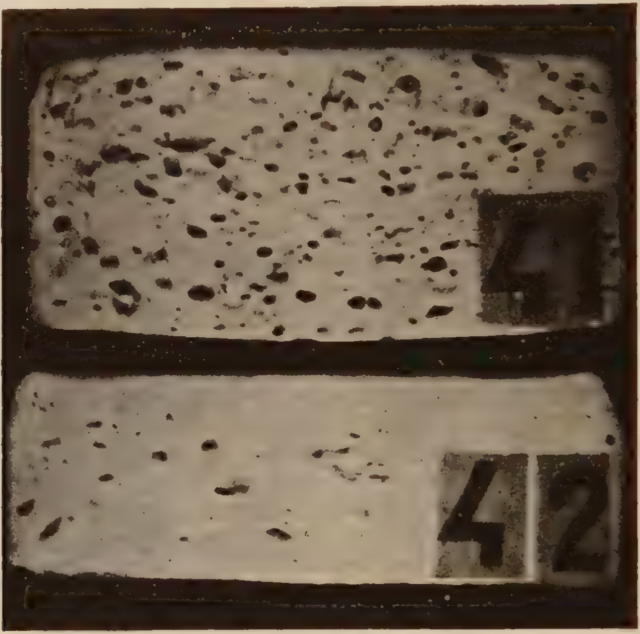


Fig. 9. The control of the early gas defect in cheese with KNO_3. Nr. 41. Milk inoculated with 1 ml culture of the blowing-positive strain 11 (A1). Nr. 42. Milk inoculated in the same way and moreover supplied with 100 g KNO_3 to 100 l milk.

We may note that both nitrate and nitrite without inhibiting the development of the blowing-positive bacteria may prevent the early gas defect. When the amount added is too small,. the bacteria after having decomposed it will anew produce gas. The use of these substances, however, entails disadvantages as nitrite will occur in the cheese acting inhibitingly on the lactic acid fermentation. So in cheeses in which the early gas defect has been succesfully combated the pH will stay on a much higher level. This is very undesirable as later on this may act unfavourably on the taste. These high pH did not occur when nitrate or nitrite had been added in too small amount, the nitrite then having disappeared before the lactic acid fermentation was at its height.

Moreover nitrate and nitrite may cause discolorations in cheese, the so called „nitrate margins" (1).

Finally these remedies have the disadvantage that the development of coli-aerogenes bacteria not being disturbed nothing can be done against the fault of unsavoury taste caused by these bacteria.

When studying the action of $KClO_3$ it directly appeared that this substance acts much more strongly on the production of gas by the blowing-positive bacteria than either nitrate or nitrite. Table 20 shows the amount of gas which coli-aerogenes bacteria could still produce from 40 ml whey after $KClO_3$ had been added. During the initial period markedly less bacteria occurred in bottles which had been provided with chlorate than in those without it. Later on these differences grew less and after 24 hours they no longer existed. At that moment a very slight production of gas set in; which was hardly noticeable in this medium.

Table 20.

Influence of $KClO_3$ on the production of gas from whey by coli-aerogenes bacteria.

Strain	$KClO_3$ added in g per l	ml gas produced after:			
		21½ h.	41 h.	46 h.	65 h.
4 (I1)	—	16.2	74.0	87.0	130.0
4 (I1)	5	0.1	1.0	1.2	2.5
11 (A1)	—	8.0	45.5	54.0	81.0
11 (A1)	5	0.1	1.4	1.6	4.0
37 (I2)	—	7.0	17.5	19.0	23.5
37 (I2)	5	0.1	0.3	0.6	0.6

The fact that coli-aerogenes bacteria after addition of 5 g $KClO_3$ still produce gas could be better demonstrated by means of lactose yeast extract with a phosphate buffer. After 2.72 g KH_2PO_4 and 5.94 g $Na_2HPO.H_2O$ had been added, the liquid thus reaching a pH of 6.9, more lactose could be fermented owing to the buffering action of these substances. Table 21 offers the result of an experiment, in which also the influence of the reduction of $KClO_3$ on the bacterial development has been determined.

Addition of $KClO_3$ acts very strongly on the production of gas and also inhibits the development of the coli-aerogenes bacteria. QUASTEL, STEPHENSON and WHETHAM (13) report that these bacteria can reduce chlorate into chlorite but that the chlorite formed brings this process to a close by its toxic action. This apparently does not happen at a concentration of 5 g per 100 l. Indeed we may conclude from the onset of the production of gas and the normal growth accompanying it, that the chlorite is reduced further to a non-toxic, not further reduceable final product, presumably KCl. As we worked with very low concentrations we could not ascertain the nature of the intermediate products and of the final one. So $KClO_3$ serves also as hydrogen acceptor in the fermentation of sugar and as such inhibits the production of gas. As the reduction gives rise to intermediate products which are toxic for the coli-aerogenes bacteria, the delay in the onset

Table 21.

Influence of $KClO_3$ on the production of gas by strain 4 (I1) from 120 ml lactose yeast extract to which phosphate buffer had been added.

$KClO_3$ added	ml gas produced after:				Number of bacteria per ml after:				
	19 h.	24 h.	43 h.	95 h.	0 h.	5 h.	14½ h.	19 h.	27 h.
—	89.0	126.0	207.0	227.0	600.000	60.000.000	130.000.000	250.000.000	300.000.000
5g/100l	0.5	3.0	30.0	57.0	600.000	6.000	2.500	30.000.000	250.000.000

of production of gas is often much lengthened. Therefore chlorate acts much more strongly than nitrate or nitrite.

In experiments with cheese we have further studied the action of $KClO_3$. Some of the results obtained are recorded in Table 22.

Table 22.

Influence of $KClO_3$ on the formation of gas in cheese.

Cheese	Strain	$KClO_3$ added in g per 100 l	After 24 hours:			mg lactose per 10 g cheese [1]
			Judgment	Number of blowing-positive bacteria	pH	
93	11 (A1)	—	5	2.500.000.000	5.45	
94	id.	1½	1—2	25.000.000	5.45	
103	11 (A1)	—	4	250.000.000	5.30	
104	id.	2	0	2.500	5.25	
97	20 (I1)	—	4	250.000.000	5.35	90.9 after 9½ hours
98	id.	2	0	60.000	5.35	85.4 after 9½ ,,
85	4 (I1)	—	4	250.000.000	5.05	
86	id.	2	0	6.000	5.05	
101	5 (I1)	—	4	25.000.000	5.30	71.2 after 9½ hours
102	id.	2	0	6.000	5.30	73.9 after 9½ ,,
99	19 (A1)	—	4	60.000.000	5.25	69.8 after 9 hours
100	id.	2	0	2.500	5.20	69.8 after 9 ,,

[1] Initially ± 200 mg is present.

The cheeses were prepared in the same way as in the experiments with nitrate and nitrite. At the beginning of curdling both lots contained equal numbers of blowing-positive bacteria. By means of 2 g $KClO_3$ per 100 l milk we can prevent the production of gas and also inhibit the development of coli-aerogenes bacteria and so combat the fermentation taste as well. The lactic acid fermentation was not affected by the supply of chlorate, as appears from the values of pH recorded in Table 22 and the estimation of lactose. The cheeses 85 and 86 are shown in fig. 10 (No. 36 = cheese 85 and No. 35 = is cheese 86).

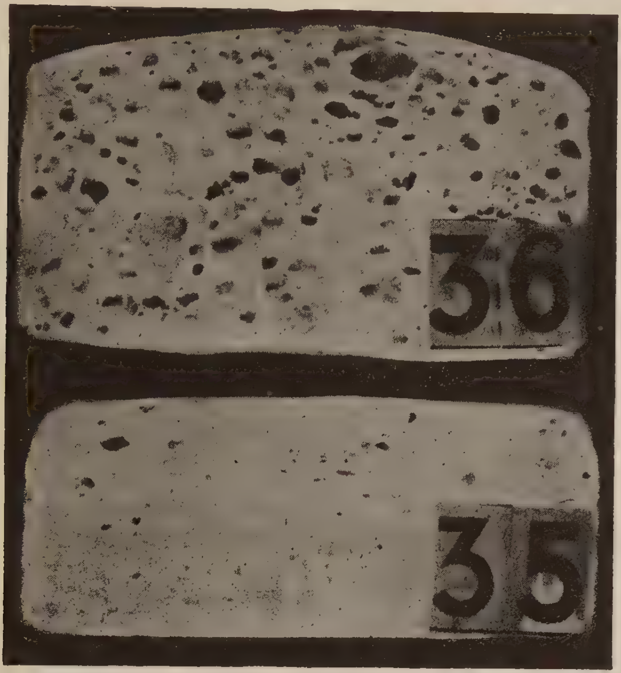


Fig. 10. The control of the early gas defect in cheese with $KClO_3$ Nr. 36. Milk inoculated with 1 ml culture of the blowing-positive strain 4 (I1). Nr. 35. Milk inoculated in the same way and moreover supplied with 2 g $KClO_3$ to 100 l milk.

As far as we could ascertain the use of chlorate did not involve any disadvantages, so that we deem $KClO_3$ the most adequate among the means of prevention of the early gas defect.

Summary.

1. In our investigation of the early gas defect in cheese we have confined ourselves to the study of the action of the coli-aerogenes bacteria.

2. We observed a marked difference in the measure in which various strains could induce this fault, although all of these produced gas in about equal amounts from lactose broth. Some strains produced much gas, other none or very little. According to this we could classify the bacteria of the coli-aerogenes group in blowing-negative and blowing-positive bacteria. As a rule the

coli types were blowing-negative, the aerogenes types blowing-positive. The intermediate types were one or the other.

3. This means that no correlation exists between the number of coli-aerogenes bacteria in milk and the measure in which the early gas defect occurs in the cheese made from it. Neither does any correlation exist between the early gas defect and the number of coli-aerogenes bacteria in cheese.

4. By means of the fermentation and curdling test it may be ascertained in a simple way whether a definite strain is blowing-negative or -positive.

5. It appeared that the deviating behaviour of the blowing-negative bacteria was caused by the citric acid in milk. Citric acid in the sugar fermentation by the blowing-negative bacteria acts as hydrogen acceptor and is converted, probably via oxalacetic acid, malic acid and fumaric acid, into succinic acid. As long as citric acid is available, these bacteria can produce no or hardly any gas from lactose.

6. The addition of citric acid to cultures of blowing-positive bacteria affected the production of gas quite differently; in fact it was increased, as these bacteria after some lapse of time proceeded to ferment citric acid with production of gas.

7. When the milk was contaminated with both blowing-negative and blowing-positive types, the cheese made therefrom as a rule was less blowing then when solely blowing-positive bacteria were present.

8. It appeared that KNO_3, KNO_2 and $KClO_3$ were able to prevent the production of gas from lactose by blowing-positive bacteria. The nitrogen of KNO_3 or KNO_2 was either assimilated or converted into NH_3. $KClO_3$ is probably reduced to KCl. As long as these reductions occurred no gas was produced from lactose. When KNO_3 or KNO_2 had been added, the blowing-positive bacteria kept on developing unremittantly. During the reduction of $KClO_3$ the development of these bacteria, however, was strongly impaired.

9. By means of these three substances we could prevent the early gas defect in cheese by adding such an amount to the milk that the production of gas by the blowing-positive bacteria was deferred up till the moment in which lactic acid bacteria could inhibit the activity of the blowing-positive bacteria. The use of KNO_3 and KNO_2, however, implied disadvantages, as the lactic acid fermentation was impaired and so the pH in the cheese remained too high. Moreover no combating of the fermentation taste caused by the coli-aerogenes bacteria could be arrived at by these means as the bacteria kept on developing. $KClO_3$, however, prevented the production of gas as well as the occurrence of the fermentation taste and the presence of $KClO_3$ did not affect the lactic acid fermentations. In our opinion among the means to prevent the early gas defect $KClO_3$ is the most adequate.

Literature.

1. F. W. J. Boekhout, W. van Dam en J. van Beynum, Verslagen Landbouwk. Onderz. **31**, 1, 1926. – 2. F. W. J. Boekhout and J. J. Ott. de Vries, Centralbl. f. Bakt. II, **12**, 89, 1904. – 3. C. R. Brewer and C. H. Werkman, Enzymologia **6**, 273, 1939. – 4. E. L. Crossley, Proc. Soc. Agr. Bact. (Gr. Br.) 1942, p. 16. – 5. W. Dorner, Landw. Jahrb. der Schweiz 1925, p. 39. – 6. Th. E. Galesloot, Over de vroeg beginnende gasvorming in kaas. Diss. Wageningen 1946. – 7. E. Haglund, E. Sandberg et Chr. Barthel, Le lait **13**, 697, 874, 1933. – 8. C. Hüttig, Molkerei Ztg. **47**, 1196, 1933. – 9. H. A. Krebs, Biochem. J. **31**, 2095, 1937. – 10. M. Mickelson and C. H. Werkman, J. of Bact. **36**, 67, 1938. – 11. Ch. Porcher, Le lait au point de vue colloidal. Lion 1929. – 12. G. W. Pucher, C. C, Sherman and H. B. Vickery, Journ. Biol. Chem. **113**, 235, 1936. – 13. J. H. Quastel, M. Stephenson and M. D. Whetham, Biochem. J. **19**, 304, 1925. – 14. H. Reynolds, B. J. Jacobsson and C. H. Werkman, J. of Bact. **34**, 15, 1937. – 15. J. Smit, Bacteriologische en Chemische onderzoekingen over de melkzuurgisting. Diss. Amsterdam 1913. – 16. H. R. Whitehead, Journ. of Dairy Res. **2**, 76, 1931. – 17. G. S. Wilson, The Bacteriological grading of milk. London 1935. – 18. A. F. Wojtkiewicz, Centralbl. f. Bakt. II, **87**, 349, 1933. – 19. M. W. Yale and J. C. Marquardt, N.Y. State Agric. Exp. Sta. Techn. Bull. 1943, p. 270.

ABSTRACTS

N. G. M. Orie, De aanwezigheid en de beteekenis van gisten in de luchtwegen. (The presence and the significance of yeasts in the air passages). Diss. Utrecht 1946.

In the nomenclature of the pathogenic yeasts the system such as it is adopted by the Centraal Bureau voor Schimmelcultures has been followed. The occurrence of these yeasts in nature and also with patients and healthy people was checked by studying the literature. Several species appear to occur on dead material as well as on the human body, although for important yeasts such as *Candida albicans* and *Torulopsis neoformans* this is not the case.

As far as the pathogenicity for animals goes it could be ascertained that *Candida albicans* (for rabbits) and *Torulopsis neoformans* (for mice and rats) possess pathogenic properties which are not explained by mere mechanical action.

A separate survey was given of those cases in which infection was limited to the lungs. There are only very few cases in which the pathogenicity of the yeast is out of doubt; more cases exist in which the yeasts may merely either play a primary or at the outset secondary part, actually, however, aggravating the case of illness in question. Next to *Candida albicans*. *Candida tropicalis* and *Trichosporon cutaneum* may play a part. Especially through the results of blood-cultures it is an incontestable fact that *Candida albicans* plays a part in human pathology and that it is not correct to regard *Torulopsis neoformans* as only agent in this respect (de Minjer). The more so as often the yeasts found in the pathological processes have been titled „*Torula*" whilst often serious mycological objections have been ignored.

Research of the author shows that in the cavity of the mouth and throat yeasts occur regularly. In blood-cultures, urines and pleurisy fluid no yeasts were found. Post mortem as a rule only a small number of yeasts were found in the alveolar tissue. The washing of the sputum, inducing the absence of throat-elements, is strictly necessary before any conclusion may be drawn as to the bacterial picture of the sputum. The presence of several bacterial (and mycotic) elements may not be considered as proof against the origin of these microorganisms out of the deeper air-passages.

An outline was given of the isolated strains of yeasts, which were isolated in the different processes. About the significance of the presence of yeasts in the deeper air-passages nothing has been established with certainty so far.

A well-defined clinical picture could not be connected therewith.

N. O.

R. Sijderius, Heterotrophe bacteriën, die thiosulfaat oxydeeren. (Heterotrophic bacteria which oxidize thiosulfate). Diss. Amsterdam 1946.

Investigations have been carried out on the heterotrophic thiosulfate oxidizing bacteria. Not only the bacteria, isolated from culture media with thiosulfate or sulfur, were able to oxidize thiosulfate, but also some bacteria not isolated from thiosulfate enrichments.

It appeared, that the capacity to oxidize thiosulfate is not a property, distributed at random over the bacteria of different genera, but the bacteria which possess this capacity, belong either to the genus *Pseudomonas* or to the genus *Micrococcus*.

The *Pseudomonas*-type (culture A) oxidizes thiosulfate to tetrathionate, so that the medium becomes alkaline,

$$2\ Na_2S_2O_3 + H_2O + O \rightarrow Na_2S_4O_6 + 2\ NaOH.$$

The *Micrococcus denitrificans*-type (culture 12) oxidizes thiosulfate to sulfate and sulfuric acid, so that the medium becomes acid. As an intermediate product sulfite is formed.

$$Na_2S_2O_3 + H_2O + 2\ O_2 \rightarrow Na_2SO_4 + H_2SO_4.$$

As it was possible to identify the heterotrophic thiosulfate oxidizing bacteria as already well-known bacteria, it is clear, that there is no reason to place these bacteria in the genus *Thiobacillus*. Only the well-known autotrophic bacteria should be reckoned to the genus *Thiobacillus*. These are *Thiobacillus thioparus* and *Thiobacillus thiooxidans* and probably also *Thiobacillus denitrificans*.

Besides the quantitative determinations of the different oxidation products of thiosulfate, by means of Warburg-manometers the quantity of oxygen needed for the oxidation of thiosulfate to tetrathionate and sulfate was also determined. The quantity thus found agreed well with the quantity calculated.

From the experiments with the Warburg-manometers it appears, that the oxidative enzymes of the bacteria of the *Pseudomonas*-type should be considered as constitutional enzymes, and those of the *Micrococcus denitrificans*-type as adaptive enzymes in the sense of Karström.

Schemes of the chemism of the oxidation of thiosulfate by a series of dehydrogenation reactions are given.

Besides thiosulfate other sulfur compounds can also be oxidized. Culture 12 is able to oxidize hydrogen sulfide and cystine, on the other hand tetrathionate and taurine were not oxidized. Sulfite was so rapidly autoxidized, that it was not possible to prove that this compound was also oxidized by the bacteria.

The *Pseudomonas*-type, as well as the *Micrococcus denitrificans*-type reduced nitrates to nitrites and nitrogen, and also often laughing-gas was produced. It should be pointed out here, that whenever nitrate is reduced to nitrogen, should also be determined whether laughing-gas is present.

Investigations have also been made, whether thiosulfate oxidizing bacteria can assimilate carbon dioxide.

As theoretically the amount of carbon dioxide which can be assimilated by the *Pseudomonas*-type is so small, that it would not be possible to detect this with the usual determination methods of carbon, I tried to settle this point by growth experiments. But notwithstanding all precautions I took to exclude every trace of organic impurities, it was not possible to demonstrate the assimilation of carbon dioxide. Since the *Micrococcus denitrificans*-type can assimilate slightly more carbon dioxide than the *Pseudomonas*-type, STARKEY could determine the assimilation of a very small amount of carbon dioxide with one of the methods for carbon determination. My growth experiments could not definitely confirm this, but there is an indication, that carbon dioxide assimilation is possible, so that it is highly probable, that these bacteria are really facultative autothrophic.

It appeared, that nitrogen compounds in the form of ammonium chloride or potassium nitrate were necessary for the growth of these bacteria.

The study of the pure cultures of strain A as well as of *Pseudomonas Stutzeri* was hampered by dissociation.

Author's summary.

F. REMPT, Onderzoekingen over het phenomeen van SHWARTZMAN. (Investigations bearing on the problem of SHWARTZMAN). Diss. Utrecht, 1946.

During our experiments, the aim of which was to come to a better insight into the mechanism of the SHWARTZMAN phenomenon, we have found a totally new phenomenon, which to our opinion can be of great value for further investigations into the nature of the SHWARTZMAN phenomenon and for the explanation of various clinical symptoms.

A rabbit which once has had a parenteral contact with an active coli filtrate, seems to be able to react differently from a rabbit which never before has had such a contact. We found two different experiments illustrating this:

1. If a rabbit, after having had some time before (10 to 56 days at any rate; possibly also a shorter or longer period) a parenteral contact with coli filtrate, receives an intracutaneous injection with coli filtrate, and if this area is clamped off 24 hours later for 4 hours, in many cases a local hemorrhage arises which cannot be distinguished from a SHWARTZMAN phenomenon. Without a former contact with the filtrate no hemorrhage appears.

2. If a rabbit, after having had some time before (one to at least 150 days) a parenteral contact with coli filtrate, and also, having been sensitized with horse serum, receives an intracutaneous injection of horse serum, almost always a phenomenon of ARTHUS arises, in the center of which in most cases arises a hemorrhage which cannot be distinguished from a SHWARTZMAN phenomenon:

a combined phenomenon of ARTHUS-SHWARTZMAN. Without the former contact with coli filtrate only a phenomenon of ARTHUS, as could be expected, was observed.

ad 1. It appeared necessary to elaborate for the experiments of this series a special technic by which it would be possible to isolate temporarily from the general circulation the skin area prepared with bacterial filtrate. We have found the solution by applying a simple clamp to the skin. Clamping the skin appeared to have an important but only accessory effect on the appearance of SHWARTMAN phenomena. Firstly: clamping the skin brings to light a state of general preparation by coli filtrate, as is described above under 1. Secondly: with this technic it has been possible to invert the order of intracutaneous and intravenous injections with bacterial filtrate, as compared with the classical way, and to prolong the time interval between these two injections considerably.

ad 2. From a group of 46 rabbits all showing a phenomenon of ARTHUS, in 41 animals combined phenomena arose, of which 27 showed complete phenomena of SHWARTMAN, 5 showed weak phenomena of SHWARTZMAN and 7 showed subphenomena of SHWARTZMAN. Of a possible relation between the size of the ARTHUS component and the size and quality of the SHWARTZMAN component of the combined phenomena, nothing could be detected. Neither did it make any difference if the time between the last contact with coli filtrate and the intracutaneous (provoking) injection with horse serum had amounted to one day or 150 days: the SHWARTZMAN phenomena occurred at random over the whole series.

The fact of a single contact with a bacterial filtrate producing such a long period of increased tendency to the formation of hemorrhages, is to our opinion of great value for the explanation of various clinical symptoms.

Author's summary.

J. VAN DER HOEDEN, ,,De Zoönosen''. H. E. Stenfert Kroese's Uitgevers-Mij N.V. Leiden, 1946.

,,In human and veterinary medicine, evidence of a certain reserve to break bounds to the ground which practice has reserved for the student of ,,the other medicine'', has been repeatedly given. Instead of occupying with that which separates, in this book the attention is directed to the natural relation existing in many respects between the diseases of man and animals''. With these words the author alleges the task he had set himself in writing this book. There is no doubt that a book like this is urgently needed. From the labyrinth of literature it is almost impossible to get an impression of the relation referred to. Both to human and veterinary surgeons the author has rendered a great service by summarizing the data from literature as well as his own investigations upon various subjects of comparative pathology in such an excellent and conveniently arranged way. A description is given of the pathogenesis, the epidemiology, the comparative clinics and the prevention of the animal diseases caused by bacteria, viruses, protozoa, moulds and yeasts, worms and arthropods, pathogenic for man. The human diseases, in which animals only serve as a virus reservoir, are discussed too. For the convenience of those who are interested in further details, many references have been gathered. This book will prove to be a manual for ready reference on the border between human and veterinary medicine. J. D. V.

(Pasteur Institute Bandoeng, Netherlands East Indies).

INVESTIGATIONS INTO RABIES II ¹)

by

L. OTTEN †

(Received December 24, 1946).

1. Results with formolized vaccine.

In her last publication the late Maria J. Otten-van Stockum ²) (13) has reported on the statistical as well as experimental results of her investigations into rabies, covering many years.

By means of an entirely new method of statistical analysis, as original in conception as simple in execution, she has shown that the usual methods of treatment seriously fall short in effectiveness. An antirabic treatment with monkey brain fixed virus, either live or killed by formaline, however, does not leave any doubt as to its effectiveness. An extensive experimental investigation furnished proof that monkey brain (10 % suspension in formaline of a concentration of 1.5 °/₀₀ and heated during 5 days at 37° C.) gives rise to a fixed virus vaccine of superior activity, thus wholly confirming the validity of her method of analysis.

For the complete records and protocols may be referred to this publication, whereas a survey of the results of all immunization experiments with rats and guinea-pigs, in as far as they have been carried out simultaneously and under equal conditions, may suffice here. As a rule the vaccinating doze was 20 mg injected in 3 steps (3, 7 and 10 mg) in the course of a fortnight, and followed 16 days later, thus 30 days after the immunization had been started, by intramuscular infection. For street virus the infecting dose was 50 mg (0.5 ml of a 10 % suspension), for fixed virus 40—50 mg (0.5 ml of a 8—10 % suspension). As a rule the number of test animals was 100 or more per experiment, grouped in series of at least 25; practically no treatment accidents occurred. The number of controls was 10 per experiment with a mortality rate of 80—100 %, in average slightly exceeding 90 %.

Table I shows distinctly that:

1. a concentration of 1.5 °/₀₀ formaline and a heating during 5

---

¹) Note of the Editors. It is a privilege to publish this important posthumous contribution of the late Prof. Otten in which he pays a well deserved tribute to the highly interesting researches and achievements of his deceased wife, Mrs. Otten-van Stockum.

²) Died May 26, 1940.

Table I.

	Formolized vaccine						Controls
1. Concentration	1.5°/₀₀		1.25°/₀₀		1°/₀₀		
	61/220 (27.7%) [1]		73/220 (33.2%)		109/220 (49.5%)		58/60 (96.6%)
2. time of heating (days)	5	1	5	1	5	1	
	41/170 (24.1%) 47/245 (19.2%)	59/170 (34.7%) —	10/50 (20%) —	21/50 (42%) —	28/75 (37.3%) —	41/75 (54.6%) 112/245 (45.6%)	68/70 (97.1%)
3. Antigenic (brain)	monkey		rabbit		sheep		
	82/400 (20.5%)		162/400 (40.5%)		158/400 (39.5%)		122/130 (93.8%)
4. Antirabic agens total monkey brain	1.5% formolized (5 days 37° C.)			1% carbolized (SEMPLE)			
	80/350 18/125		(22.8%) (14.4%)	150/350 45/125		(42.2%) (36.0%)	58/60 (96.6%)

---

[1] To be read: after infection 61 of the 220 immunized rabbits died of rabies (= 27.7% mortality).

days at 37° C. provides for the best results. The difference in rate of mortality induced by the concentration of 1.5 and of 1 °/₀₀ amounts to 21.8 %, far in excess of the average deviation (σ 4.5); between the groups of 1.25 and 1 °/₀₀ formalin the difference is significant as well (16.3 % σ 4.6). The difference owing to the period of heating is also striking: in the largest group with 1.5 °/₀₀ concentration it exceeds if not the average deviation (10.6 % σ 4.9) then the probable deviation (10.6 % ± 3.3). The difference between both extreme groups which are acted upon both by concentration and by period of heating is of course the most pronounced (26.4 % σ 4.1).

2. Monkey brain furnishes a much more active antigen than rabbit or sheep brain; the rate of mortality is reduced to nearly half (difference 20 and 19 % respectively σ 3.1).

3. The formalized vaccine gives better results than the 1 % carbolized vaccine of SEMPLE, more especially when monkey brain is used; the mortality the former entails is even 2½ times lower (21.6 % σ 5.3).

The results of antirabic treatment in man agree herewith. An experiment with carbolized vaccine in early treatment (in the first week after the bite when best results may be expected) covering the years 1922—30 produced among 12 cases of rabies no less than 4 „failures", *viz.*, cases of rabies, in which the symptoms set in more than 30 days after the treatment has been started. In the course of more than 25 years among 52 cases of rabies treated in the first week with the live and later with the formolized vaccine not a single „failure" has been recorded. The fact that also with the monkey brain fixed virus, live as well as formolized, a result may be attained which well may be claimed maximal appears from Table II, recording the results of the preventive treatment in man (Indonesians in the first week after the bite) with various, successively applied methods.

Table II.

Method of treatment	number of treated	number of cases of rabies			mortality	"failures"
		total	— 30 days after bite	> 30 days after bite		
Dry cord rabbit (attenuated) 1895—1905	1.398	65	30	35	4.6%	31 = 48%
Rabbit brain (live) 1906 — medio 1916	3.167	92	53	39	2.9%	28 = 30%
Monkey brain (live) medio 1916—1932	2.753	33	32	1	1.2%	0%
Monkey brain (formolized vaccine) 1930—1941	3.108	19	18	1	0.6%	0%

As Maria J. Otten-van Stockum in the paper quoted and in her treatise of 1935 (12) has fully pointed out, the rate of mortality from rabies during or after treatment as such can never be a reliable measure for the effectiveness of the method followed. So it is not the strong reduction in mortality but the reduction in the occurrence of „failures" which has chiefly to be pointed to. Not a low rate of mortality but the complete absence of „failures" furnishes proof for the outstanding quality of the antigen used since 1916, the monkey brain fixed virus. Even in delayed treatment, up to the 3rd–4th week after the bite, the number of „failures" remains extremely low: on a total number of 10.069 Indonesians which had been treated with this fixed virus, live or dead, 132 cases of rabies occurred with merely 2 „failures" (both having been attended to in the second week) *e.g.*, not more than 1.5 %.

This table also shows that the treatment with formolized monkey brain virus does not by any means lag behind that with live virus, corroborating the results of the alternating experiments in 1930-38. Not a single „failure" had occurred then and the rate of mortality in the group treated with dead fixed virus (4.626) was even less than half that of the group treated with live virus (9.621). As a matter of fact the vaccinating dose of the formolized vaccine was higher than the dose of the live vaccine, 1120–1520 mg and 470 mg respectively, but even this maximum dose of about 1.5 g may be claimed as very low, when compared with the doses of dead vaccine such as they are administered in other modes of treatment and which in some Institutes may come up to 8—10 g and even more.

Next to a difference in the rate of mortality between both methods of treatment closer investigation also reveals a difference in the period of incubation. This appears from Table III recording the average periods of incubation for all cases of rabies when treated with live virus (Högyes-method). covering 1916-37 and those treated with dead virus (formolized vaccine) covering 1930-41. The cases have been grouped according to the position of the wound and those having been attended to in the first week after the bite have been inserted separately in brackets.

Table III.

Position of wound	Average incubation			
	when treated with monkey brain fixed virus			
	live		dead	
	number	incubation	number	incubation
head	39 (20)	24.0 (21.6)	25 (12)	22.4 (19.4)
arms	27 (7)	32.2 (25.2)	8 (3)	26.7 (22.5)
legs	19 (6)	30.2 (26.5)	11 (4)	27.0 (21.5)
Total	85 (33)	28.0 (23.2)	44 (19)	24.2 (20.2)

In the treatment with dead virus the incubation period appears to be shortened for some days for wounds of the head as well as of the limbs [1]). This does not in the least agree with the conclusion, drawn by Nitsch (10), based on the shorter incubation periods of treated cases when compared with untreated, who assumes that the reduction in the incubation period would be owing to the treatment with live although attenuated virus. That he is entirely in the wrong and that this shifting of the incubation period is the unevitable result of the inverse selection of the group of the treated in respect to the group of the untreated has been pointed out already by Maria J. Otten-van Stockum in her monography.

This does not mean that even now this view has been completely abandoned; in fact Proca and Bobes (14) have claimed in 1940 that it might be conceived, that live virus would accelerate the course of rabies infection [2]). The risk that street virus would more easily arrive at a general dessimination under the influence of fixed virus, was even deemed so serious that in Boecarest in 1936 an attenuation of the treatment was introduced (by omitting cord of 1—0 days drying).

---

[1]) In contradistinction to the longer incubation which is generally noted for wounds of the head when compared with those of the arms, the above figures show practically no difference; the fact, that cases with longer incubation are prevented owing to the effective treatment, may perhaps explain this.

[2]) ,,might not impossibly accelerate the course of the rabies infection"

In consequence the number of cases with a short period of incubation actually decreased from 70—80 % to 10 %, but on the other hand the rate of mortality appeared nearly doubled when compared with that in two preceding years and the number of ,,failures'' increased up to 60—70 %!

The opposite happened in Tonkin, where GENEVRAY and DODÉRO (3) after reinforcement of the Pasteur treatment (by the introduction of a period of drying of 1—0 days) could report a reduction of the rate of mortality of ± 50 % along with a reduction in the number of ,,failures'' to less than half. In 1926 to medio 1929 among 2440 treated 24 cases of rabies occurred, among which 6 ,,failures'' (25 %), in the succeeding 3½ years this number amounting to 17 out 3523 cases, among which 2 ,,failures'' (11.8 %). Meanwhile DODÉRO (2) makes the same mistake when, based on a comparison of the incubation periods between treated and untreated, he concludes that the antirabic treatment would shorten the period of incubation. Apart from the fact that he worked with incomparable groups this conclusion is surprising as not merely the number of ,,failures'' but the total mortality markedly decreased. When a reinforced treatment with live c.q. attenuated virus would actually lead to an earlier onset of the disease, it might be expected that such a harmful influence would lead to the activating of the infection process in a number of cases where otherwise it would not have developed, in other words far from a marked reduction in the number of cases of rabies an increase in mortality would have to be expected.

Moreover the view adhered to by the latter authors is quite contrary to the findings in experimental immunisation against bacterial infection. However acceptable the occurrence of a negative phase may sound theoretically, rather the contrary appears in vivo, for indeed test animals in active immunization, irrespective of the nature of the vaccine live or dead, far from displaying a weakened resistance, will as a rule prove to possess from the very first days an increased resistance against injection, revealing itself by a reduced rate of mortality along with a lengthened period of life when compared with the untreated controls. When an antigen low in activity has been used, this lengthening of the period of life, c.q. of illness, is often the only, be it weak, symptom of the existence of immunity.

Similar data bearing on immunization by invisible viruses, more especially by fixed virus are not known to me. From post-infectional immunization, such as it is induced by the treatment in man, hardly any experimental success may be expected, at least not when small test animals such as rabbits or guinea-pigs are used. Having classed my own material in as far as this bears on the immunization with live fixed virus, the period of incubation in rabbits died from infection with street virus appeared to be averagely 2.5 days longer than that of the comparable controls. Although in these experiments the immunization had occurred prae-infectionally, a certain analogy with a negative phase exists. In fact as the result of the treatment nearly 1/3 of the animals died from an infection with fixed virus with an average period of life of 18 days (min. 13—max. 23), thus during the last days or even after the period of immunization. So the survivors on the date of infection thus might be expected to have hardly recovered from a serious infection with fixed virus and many of them would have had to stand the virus infection rather with a weakened than with a strengthened resistence. Still the rate of mortality, although high (33 %), was three times lower than that of the controls (90 %) and was accompanied by the lengthening of the period of incubation mentioned above; in the rabbits immunized with fresh formolized vaccine without any treatment accident a lengthening of the period of incubation, with 3.3 days was recorded, thus only 0.8 day longer, although the mortality was merely 9.1 %.

Be this as it may, the mere observation that the average period of incubation in cases of rabies treated with formolized vaccine shows a definite shortening when compared with those treated with live vaccine sufficiently disproves the assertion that this shortening should be ascribed to the action of the live fixed virus. Thus is left merely the question as how to explain the shortening of the period of incubation: whether this phenomenon might be claimed the proof of a still more efficient immunization in such a sense, that not only all cases are saved in which the onset of illness would have occurred more than 30 days after the commencement of treatment, but also part of the cases with a still shorter incubation is spared.

In her investigations on rabies MARIA J. OTTEN-VAN STOCKUM has assumed an ideal method of treatment by means of which the gradually developing immunity attains its height 30 days after commencement of the treatments and can then prevent any further cases of rabies, „failures", irrespective of the moment the treatment has been started. The maximal period of incubation for this hypothetical treatment, when started in the first or the second week after the bite, was calculated to 36 or 43 days respectively, whereas the longest period of incubation observed in the treatment with live monkey brain fixed virus in the first two weeks after the bite amounted to 32 and 38 days respectively, thus 4 and 5 days shorter. This led her to the suggestion that a still further reduction of the rate of mortality, *viz.* within the 30 days mentioned, might be possible.

It appears striking that the longest period of incubation recorded nowadays in the treatment with formolized vaccine in the first two weeks after the bite amounts to 27 and 35 days respectively, thus markedly lower than the calculated limit. Whether actually still more can be attained at with the formolized vaccine than what was claimed possible by MARIA J. OTTEN-VAN STOCKUM may only come to light, when a more extensive material will be available.

Although among the European moiety of the population rabies is much less frequent, for the sake of completeness in a synoptical table may be recorded the total number of persons, Europeans (Eurasions) as well as Indonesians, who have been treated since the foundation of the Institute Pasteur (cf. Table IV).

As between both groups no difference has been made, except that for the European group as a rule a lower dosage has been chosen, it is obvious that the remarkedly lower rate of mortality

Table IV.

Period	Europeans				Indonesians			
	number		mortality	contact cases	number		mortality	contact cases
	treated	cases of rabies			treated	cases of rabies		
95–1900	660	6	0.9 %	—	915	37	4.0%	—
01–1910	1382	3	0.2 %	28.0%	2815	94	3.3%	1.7%
11–1920	1932	5	0.2 %	42.5%	3931	78	1.9%	3.6%
21–1930	1478	1	0.07%	48.5%	4213	49	1.1%	3.4%
31–1940	498	0	0 %	28.0%	5378	59	1.1%	1.6%

in this group cannot be explained by any better effect of treatment in Europeans. It is simply the result of the fact that the risk of infection is much less. Not only that wounds in the head, the most dangerous cases, are unfrequent, but the wounds are mostly of a less dangerous nature, as the number of contact cases, in which the risk of infection falls practically to zero, proves. Among the Indonesians these contact cases amount to hardly 2.5 % of the total, whereas in the European group they amount to 39 %, in 1921-30 even to nearly 50 %.

Be this as it may even in the latter group the improvement in efficiency of the successive methods of treatment can be clearly recognized:

1. Pasteur-method (1895-1905): 9/1421 (mortality 0.56 %), among which 7 ,,failures'' (77.7 %).
2. Högyes-method: rabbit brains (1906-medio 1916): 4/1610 (mortality 0.25 %), among which 1 ,,failure'' (25 %).
3. Monkey brain fixed virus, live and dead (medio 1916-1940): 2/2919 (mortality 0.07 %), among which 0 ,,failures''.

Although the mortality in treatment with monkey brain fixed virus is very low, I shall refrain from drawing any conclusion as to the results obtained in Europe with different methods of treatment. Although ,,failures'' are lacking, the material is too scanty, the number of factors influencing the risk of infection too incalculable to allow of any comparison. My detailed critical examination of the method followed by Mc. Kendrick (9) in his statistical analysis of all rabies material may throw further light on this [1]).

As to the duration of treatment which amounted to a fortnight for slight wounds, to 3 weeks for severe wounds and for belated cases, Maria J. Otten-van Stockum could not ascertain any marked difference in the content of rabicide substances, by means of serological tests in a number of bitten persons, part of whom had been treated during 14 days, part during 21 days. It is true that after a short treatment the initial increase in rabicide activity of the serum lagged behind that obtained after a longer one, but after 30 days the rabicide activity, irrespective of the duration of treatment, appeared to have reached a same level. Therefore it was not possible on the base of this small difference to settle the point whether the fortnightly treatment would lag behind that of 3 weeks, the less so as the content of rabicide antibodies may not be claimed a wholly reliable measure of the degree of immunization actually existing.

---

[1]) See postscript on the first part of the publication of Maria J. Otten-van Stockum (13).

So for settling experimentally in how far a shortening of the duration of treatment, desirable in many respects, would be justified, only one more method was available, *viz.*, the alternating treatment. The results of such a treatment which was carried out in 1939-42 and remained confined to those cases which had been attended to no later than 14 days after the bite, are recorded.

Table V.

Attended to	period of treatment	
	2 weeks	3 weeks
in 1st week after bite	3/580	2/558
in 2nd „ „ „	4/267	5/276
total	7/847	7/834

Thus in a two weeks' treatment as many cases of rabies occurred as in a 3 weeks' one. The localization of the wounds was also nearly equal: 4 in head, 1 in arm and 2 in legs in the first group, 4 in head, 1 in leg and 2 in arms in the second group. The material is still too scarce to allow of a definite conclusion, the less so as merely 2 out of 7 cases had been subjected to a complete treatment. Still the similarity in results and most of all the lacking of ,,failures'' even in a short treatment is such that it may be claimed encouraging.

2. KEEPING QUALITY OF THE FORMOLIZED VACCINE.

As in course of time the formolized vaccine appeared to answer the expectations, it was obvious, that the possibility of centralization had to be studied. Although for many reasons it may be preferable that not merely the preparation, but also the antirabic treatment take place in a Central Institute under expert supervision, one great objection may be raised, *viz.*, the delay in treatment and this the more so in an archipelago with limited means of speed traffic. Even leaving alone the high costs of travelling and residence, this drawback is so serious that decentralization is indicated as soon as a reliable vaccine of a satisfactory keeping quality is available.

So first of all it appeared indicated to ascertain in how far activity kept pace with keeping quality of the formolized vaccine, *e.g.*, whether it would keep active when ready for use as a 2 % suspension it was kept at room temperature, c.q. in cold storage during some months. In order to settle this point, from monkey brain fixed virus (prepared as usual as a 33 % suspension in 70 % glycerol at 12—15° C. and kept stored below 0° C.) a 10 % formolized vaccine was prepared. Periodically, as a rule 3, 2 and 1 month before the commencement of the immunization experiments, dilutions into 2 % suspensions were made, which were kept stored at room temperature (22° C.) c.q. in cold storage at 5° C. In order to check the fixed virus in hands to its activity a 10 % formolized vaccine freshly prepared from the concentrated suspension (after dilution into 2 % suspension) was tested out.

All in all 7 experiments with rabbits have been carried out, exactly following the technique applied by Maria J. Otten-van Stockum. Per sample of vaccine 20—25 rabbits have been used, as checks at most 10, which number sufficed owing to the strong virulence of the infecting street virus.

Although in fact the use of street virus for the testing of the antigenic value of the antirabic vaccine by means of intramuscular infection is preferable to the use of fixed virus this is mostly abstained from, it being claimed that the virulence of street virus would not only be variable, but also weaken rapidly.

It is perfectly true that in an initial test the virulence of street virus may vary greatly, but this depends mostly on the condition of the brain matter when injected. A slight putrefaction may suffice for weakening strongly the virulence of the virus. Moreover tests on virulence of this nature have often been confined to very few animals (1—2), a fact which owing to the widely divergating individual resistance may lead to very misleading results. However, when a number of at least 10 animals is injected intramuscularly with fresh brain matter, in most cases a mortality of 80—100 % will be reached. The virulence of street virus which in moderate dosage (0.5 ml of 10 % suspension = 50 mg) meets these demands may certainly be qualified as quite satisfactory.

In a systematic investigation, carried out in 1940-42 of fresh Negri-positive material, mostly derived from Bandoeng and surroundings, 25 out of 32 day brains, thus over 75 %, yielded this result. In 32 series of 10 rabbits the mortality amounted to 2 × 60 %, 5 × 70 %, 13 × 80 %, 7 × 90 % and 5 × 100 %, averaging 82.5 %; the shorted period of incubation, or rather term of life, amounted to 10 days, the longest to 30, the average 18.3. Although its weight is merely 1/3 of that of the rabbits, the guinea-pig in this respect appears to be slightly less susceptible; among 32 series which had been put up simultaneously with those of the rabbits and thus were wholly comparable, the number meeting the above demand was about 65 % (21) of the total (3 × 60 %, 8 × 70 %, 9 × 80 %, 9 × 90 % and 3 × 100 %, averagely 77.5 % mortality). The duration of life varied from 9 to 37 days, averagely 19.4.

The second objection against the use of street virus is also unfounded, as long as an adequate storage is provided for. When the street virus prepared as has been described for fixed virus is kept stored as a 33 % suspension in 70 % glycerol at 12—15° C. below 0° C., it can keep up the same virulence during several months and even several years, at least invariably yield equal results when administered in a dosage of 50 mg intramuscularly.

As an example may be cited street virus no. 44/40 used in most of my experiments which in the initial test (29-12-1940) had yielded 100 % mortality in a period of 20—29, on the average 24.5 days. Among 15 series of

10 rabbits it induced practically constantly a mortality of 100 % within the first month; one rabbit survived the infection and only once death supervened after 34 days. In the final lot of 10 rabbits inoculated nearly 2 years later (10-12-1942) all died in the course of 13—21, on the average 15.9 days. 5 series of guinea-pigs, infected in the course of the same period, yielded about equal results: a mortality of 90—100 % (47/50 = 94 %) in the course of 10—29, on the average 18.4 days.

Another street virus, no. 11/38, which had been in use by MARIA J. OTTEN-VAN STOCKUM during 2½ years, in 14 series of 10 rabies yielded a mortality of 90—100 % (138/140 = 98.5 %) with a duration of life of 11—40 days, averagely 18.1; in 6 rabbits, e.g. not yet 5 %, this amounted to more than 30 days. Among 40 series of guinea-pigs inoculated with this virus along with the rabbits, mortality reached from 70 (1 ×)—100 % (369/400 = 92.2%) with a period of life of 9—53 days, averaging 19.7; a larger number of guinea-pigs survived the infection more than a month, *viz.*, 46 (12.4 %).

In the following schedule the results are recorded synoptically obtained with these two street viruses along with those of a few other ones having been tested in the same way during a shorter period and offering the same picture (cf. Table VI).

As a matter of fact it is plausible that in regions, where hydrophobia occurs merely sporadically, the street virus may be of lower virulence than in Java, where the epizoötic occurs endemically and ever and anon gives rise to local epidemics. All the same it will be possible, provided none but fresh material will be applied and adequate conditions of storage are kept up, to obtain regular results, be it perhaps by means of a higher dosage. Preliminary tests will then be unavoidable but the amount of works and costs involved will surely be counterbalanced by the primary importance of the use of intramuscular injection with street virus in tests of active immunization, which gives the nearest approximation to the mode of transmission such as it occurs under natural conditions.

The vaccinating dose amounted to 20 mg injected in three steps (3, 7 and 10 mg) in the course of a fortnight, 16 days later thus 30 days after commencement of the immunization followed by intramuscular injection with street virus in the dose of 50 mg. As after some experiments it appeared that a 2 % suspension even when stored at 50° C. decreased fairly rapidly in activity, also the 10 % suspension was tested. In the synoptic table VII results of all experiments are recorded; merely one out of 70 controls survived the injection.

Owing to the great number of vaccine samples which had to be tested simultaneously, it was not possible to run a complete comparative test along with each experiment. None the less all the experiments show clearly that the formolized vaccine with increasing age decreases in activity. This is most pronounced at room temperature, but even at 5° C. the decrease is unmistakable in the 2 % suspension and, although in a lesser degree,in the 10 %. More especially at room temperature the results with a same sample of vaccine divergate markedly; so the mortality caused by a 2 % suspension, having been kept stored at this temperature during 3 months, varies between 20 and 68 %, by another, having been kept stored during 2 months, between 10 and 52 %. This will have to be ascribed primarily to a difference in quality of the

number street-virus	period of investigation	rabbit						guinea-pig					
		mortality			period of life			mortality			period of life		
		course	%	average	period	average	> 30 days	course	%	average	period	average	30 days
44/40	2 years	149/150	90–100	99.3	10–34	18.1	1=0.67%	47/50	90–100	94.0	10–29	18.4	0
11/38	2½ year	139/140	90–100	98.5	11–40	18.1	6=4.35%	369/400	70–100	92.2	9–53	19.7	46=12.4%
1/36	1½ year	45/50	80–100	90.0	8–40	17.8	3=6.6 %	85/90	80–100	94.4	14–53	20.6	9=10.6%
4/42	1 year	37/40	70–100	92.5	11–28	21.1	0	37/40	70–100	92.5	11–26	16.9	0
18/42	10 months	37/40	80–100	92.5	11–24	17.3	0	39/40	90–100	97.5	13–27	17.8	0

Table VII.

Exp. no.	formolized-vaccine, 2% suspension								fresh	controls
	22° C.				5° C.					
	6 m	3 m	2 m	1 m	6 m	3 m	2 m	1 m		
1	—	17/25	13/25	8/25	—	7/25	—	—	5/25.	9/10
2	18/25	15/25	11/25	7/25	5/25	—	—	—	4/25	10/10
3	—	—	—	—	7/20	5/20	2/20	1/20	1/20	10/10
	5° C.									
	2% suspension				10% suspension					
4	—	7/20	5/20	3/20	—	4/20	3/20	2/20	2/20	10/10
5	—	—	—	—	—	4/20	3/20	2/20	2/20	10/10
6	—	—	—	—	—	2/20	2/20	2/20	1/20	10/10
7	—	3/20	2/20	1/20	—	1/20	0/20	0/20		
	22° C.									
	—	4/20	2/20	1/20	—	2/20	1/20	1/20	0/20	10/10

antigens in question as even in series treated with freshly prepared vaccine mortality varied from 0—20 %[1]).

A perfectly reliable evaluation of the keeping quality of the formolized vaccine can only be arrived at by means of a simultaneous testing of the samples of vaccine, rendering the rates of mortality wholly comparable; such data are inserted in Table VIII; the total amount of rabbits treated with fresh formolized vaccine being lower than those inserted in the other colums, will not need any further explanation.

Table VIII shows distinctly that:

1. The activity of the formolized vaccine, irrespective of the concentration of the suspension and of the temperature of storage, gradually decreases in the course of months. The difference in mortality between both extreme groups, that of fresh and 3 months old vaccine, is greatest, but a marked difference exists also between the group of 1 and of 3 months (16.6 % σ 3.9).
2. The activity of the formolized vaccine irrespective of concentration is kept up better at 5° C. than at room temperature, the difference in total mortality amounting to no less than 15.8 %, σ 4.2.
3. The activity of the formolized vaccine is better kept up as a 10 % suspension than as a 2 % dito both at room temperature and at 5° C. The difference in total mortality amounted to 77.7 % σ 3.5 and is thus within the average deviation, but outside the probable error (7.7 % ± 2.2).

Although in one respect this investigation was disappointing, in as much as the formolized vaccine, when stored as 2 % suspension ready for use at room temperature, appeared to be of a lesser keeping quality than formolized vaccines use to be, in another respect it furnished proof that the 10 % suspension when stored at 5° C. decreased very slightly in activity. So it appears perfectly warranted for antirabic treatment to send out the vaccine in this shape, provided adequate precautions as to temperature have been taken, for use after dilution into 2 % within the period mentioned. As aeroplanes have developed into a general means of traffic and the ice-box at least in the tropics may be considered a regular kitchen ustensil, the objections against forwarding and storage are of no weight when compared with the preponderant objection involved in delay of treatment. As in Java the speed traffic has already developed satisfactorily, it seems to me that here centralization is not immediately needed. The need for it is doubtlessly

---

[1]) In the rabies department according to need (usually weekly) part of the concentrated (33 %) fixed virus suspension (always a mixture of brain of 3 monkeys) is worked up into 10 % formolized vaccine. The daily amount needed is diluted into 2 % suspension and kept ready for use (4 ml = 8.0 mg). As for the successive experiments, the interval between which covered 3—6 months, the 33 % suspension then available for the antirabic treatment was used which is used up in 1½—2 months, these immunization experiments have involved 7 lots of fixed virus suspensions.

Table VIII.

Classing	formolized-vaccine kept during ..... months			
1	3	2	1	fresh
2% — suspension at 22° C. 2% — ,, ,, 5° C. 10% — ,, ,, 5° C.	36/70 15/60 11/80	26/70 9/60 8/80	16/70 5/60 8/80	9/70 3/60 5/80
total	62/210 (29.5%)	43/210 (20.5%)	27/210 (12.9%)	15/150 (10%)
	kept at . . . during 3—6 months			
2	22° C.		5° C.	
2% — suspension 10% — ,,	42/110 4/60		18/110 1/60	
total	46/170 (27.0%)		19/170 (11.2%)	
	kept as . . . during 1—3 months			
3	2% suspension		10% — suspension	
at 22° C. at 5° C.	7/60 21/120		4/60 10/120	
total	28/180 (15.5%)		14/180 (7.7%)	

most urgent in Celebes (Makassar), next to Java the centre of hydrophobia in Netherlands East Indies.

3. CHLOROFORMIZED VACCINE.

Since 10 years in U.S.A. the chloroformized vaccine has taken its place next to carbolized vaccine, which at least in veterinary practice it may come to substitute, when further investigation might confirm the unfavourable results as to the activity of the carbolized vaccine (WEBSTER (17, 18, 19), HABEL (4), WYCKOFF (20, 21)). Up till now communications about chloroformized vaccine are few and especially parallel investigations with carbolized vaccine have scarcely been carried out.

KELSER (7) confined himself to 5 immunization experiments with 1 % chloroformized vaccine in a small number of rabbits; 53 % (16/34) appeared resistant against subdural infection, whereas not a single check survived (8/8) and 91 % (2/24) against intramuscular infection, 30 % (7/10) of the checks surviving. KELSER reports to have reproduced these results several times; however, in these tests a fairly weak intramuscular infection was involved, considering the mortality of the checks which varied from 40—80 %. Moreover the vaccinating dose amounted to 2—4 ml of a 33 % suspension (660—1330 mg), for a rabbit a very high dosage. The short report finishes with the suggestion that in dogs a dose of 5 ml might appear active against natural infection.

SCHOENING (16) reports that in 2 experiments with chloroformized vaccine after intramuscular infection with street virus all dogs survived (0/9 checks 11/14), whereas not yet 60 % (5/12 checks 9/11) survival could be obtained with carbolized vaccine. There is, however, only one experiment in which both vaccines have been tested simultaneously, resulting in no mortality with the chloroformized vaccine (0/4) and 30 % (3/10) with the carbolized vaccine. But even this experiment can hardly be claimed comparative as two different samples of carbolized vaccine have been used, the one yielding 100 % (0/5) survivals, the other 40 % (3/5). Besides the dosage of the carbolized vaccine as a rule was lower and half of the dogs treated herewith were injected at a 10 days later date. Although the results of these experiments may hardly be claimed convincing, SCHOENING claims that according to the data available, chloroformized vaccine is superior to the carbolized vaccine, be it under the restriction that the drawing of a final conclusion has to be postponed till practical results will be available.

BARNES, METCALFE, LENTZ and MARTINDALE (1) report on an experiment with chloroformized vaccine in dogs, 35 % (7/20) of the dogs dying off against 55 % (11/20) of the checks, at first sight thus a better result than the one obtained in 1930 with carbolized vaccine in which a mortality of 89 % (24/27) against 91 % (10/11) [1] of the checks was reported. The total amount, however, also contains the results of intramuscular and intravenal injection, amounting to 0/7 and 0/3 in the vaccinated dogs and 1/7 and 0/3 in the checks. When these two groups with an extremely weak infection are discarded and merely the subarachnoidal infection is taken into account, the result is 70 % mortality among the vaccinated dogs (7/10) against 100 % (10/10) of the checks. This is a relatively good result considering the mode of infection and its severity but all in all we are here concerned with a single experiment and a single sample of vaccine. So BARNES *et al.* conclude very prudently that perhaps this vaccine might have some effect upon certain strains of street virus.

---

[1] BARNES takes the reinfections also into account and discards the intravenous infection and that by means of bite, because here all checks

REICHEL and SCHNEIDER (15) carried out some experiments with dogs and rabbits in which vaccines prepared in various ways have been used. The only experiment in which chloroformized vaccine has been compared to a carbolized one concerns a single test with two lots of 3 rabbits. Among the rabbits treated with formolized vaccine merely one survived the intralingual infection with fixed virus; the checks gave a similar result whilst the 3 rabbits immunized with carbolized vaccine survived.

WEBSTER (17, 18, 19) obtained entirely negative results in mice with carbolized as well as with chloroformized vaccine; he reports that the chloroformized vaccine in intraperitoneal immunization as a rule is effective, carbolized vaccine only rarely. When tested on puppies the chloroformized vaccine appeared to be more active, in 4 experiments in as far as these bore on a comparative investigation, 58 % mortality (14/24) occurred whilst the carbolized vaccine gave 81 % (31/38) mortality against 88 % (22/25) in checks. In connection with this relatively high mortality a 5th experiment with a higher dosage of the chloroformized vaccine has been put up, partly intraperitoneally (2 × 5, 10 or 20 ml), partly subcutaneously (2 × 5 of 10 ml); with the former method of immunization the mortality after infection amounted to 23 % (3/13), with the latter 50 % (4/8) against 100 % of the checks (4/4). Here, however, no carbolized vaccine has been tested.

Although there is a considerable difference in mortality (23 %) in the first 4 experiments, when the tables are inspected more closely, it appears that next to 6 carbolized vaccines only 2 chloroformized ones have been tested, whereas only chloroformized vaccine No. 1 and carbolized vaccine No. 9 have been put up simultaneously, although in small lots and not even in equal numbers. Next to this merely two single lots are comparable (see Table IX).

So the difference in mortality induced with a partly weak, partly reinforced intramuscular infection with street virus after im-

---

survived. The same, however, keeps true for the intra-ocular infection, at least for the initial one and so it seems to me more justified here to take into account merely the sub-arachnoidally infected dogs. These numbered 23.7 of which survived the first infection, thus 30 % against 20 % of the checks (8/10), a somewhat larger difference but still of low significance.

Table IX.

vaccine	result	vaccine	result	controls
Chloroformized no. 1	1/4	carbolized no. 9	4/5	5/5 (2 d.l.c.)
,,	4/4	,,	2/3	2/4 (20 d.l.m.)
,,	2/5	,,	1/3	1/3 (2 d.l.m.)
	7/13 (54%)	—	7/11 (64%)	8/12 (66%)
Chloroformized no. 1	2/5	carbolized no. 1	4/5	4/5 (2 d.l.m.)
,, ,, 3	5/6	,, ,, 3	2/3	6/6 (1 d.l.c.)
total	14/24 (58%)	total	13/19 (68%)	18/23 (78%)

munization with either chloroformized vaccine No. 1 or carbolized vaccine No. 3 appears not to exceed 10 %; the sum total of all the comparable experiments yields the same small difference. It has moreover to be kept in mind that in these experiments an equal volume (5 ml) has been administered, although the carbolized vaccine is employed in a 33 % suspension, whilst carbolized vaccine as a rule contains a suspension of not over 10 %. WEBSTER had in view the testing of the activity of these commercially supplied vaccines according to the dosage indicated [1]). It is clear, however, that even if these tests had furnished proof of the superiority of chloroformized vaccine as a commercial product, this would by no means have proved that chloroform as antirabic agent would yield a vaccine of higher antigenic value than the vaccine obtained by means of carbolization, a fact which would be of far greater importance scientifically. All in all it seems to me that the results of his experiments do not corroborate the preference WEBSTER claims for the chloroformized vaccine.

JOHNSON and LEACH (5, 6) have carried out a great many tests in dogs with carbolized and chloroformized vaccine derived from one and the same firm: the carbolized vaccine as a 20 % suspension of sheep brain, the chloroformized vaccine as a 33 % suspension of calf cord and brain. Of both vaccines several samples varying in age were tested: the vaccinating dose of 5 ml, administered either subcutaneously or intraperitoneally was followed after 28—41 days by intramuscular infection with 100 mg street virus. The results of these tests as far as they are mutually comparable are collected in Table X; the total results are also inserted.

---

[1]) HABEL (4) followed another method in the comparative investigation of a great many carbolized vaccines: he determined the d.l.m. (a mortality of over [illegible] % after intracerebral infection in mice) of the strains of fixed virus employed in the preparation of these vaccines and immunized with a dose corresponding to 400 d.l.m. of the strain tested. Apart from the fact that this d.l.m. is hardly an exact unity, it seems to me that the relation between virulence and antigenic value is not narrow enough to warrant its use as a measure of equivalency in dosage.

Table X.

Exp. no.	carbolized vaccine subcutaneously	carbolized vaccine intraperitonially	chloroformized vaccine subcutaneously	controls
1—9	20/76	—	—	49/82
0—13	6/29 (20.7%)	10/29 (34.5%)	—	17/38 (40.7%)
4—15	—	9/23 (39.1%)	0/22 (0 %)	17/25 (68.0%)
6—17	—	—	2/28	17/30
otal	26/105 (24.7%)	19/52 (36.5%)	2/50 (4.0%)	100/175 (57.1%)

So Johnson and Leach in contradistinction with Webster report fairly satisfactory results from subcutaneous immunization with carbolized vaccine (20.7 resp. 24.7 % mortality), even better than from intraperitoneal immunization (34.5 resp. 36.5 % mortality), subcutaneous immunization with the chloroformized vaccine yielding the best results. It is to be deplored that the activity of both vaccines when administered subcutaneously has not in a single experiment been compared simultaneously. Moreover it has to be kept in mind that here as well the dose of the chloroformized vaccine exceeded that of the carbolized vaccine by more than half.

Johnson and Leach ascribe the great difference in activity to the fact that the heat sensitive fixed virus during its preparation into carbolized vaccine is kept exposed for 3—24 hours to 37° C. They presume that the antigenic matter is narrowly connected with live virus and so assume that fixed virus no matter how strongly attenuated can only be active when alive. Still many adhere to this view: Lépine and Sautter (8) inter alia assume that the action of carbolic acid on the fixed virus leads to a „complex stable", which leads to a complete loss of virulence within a few days in this sense that the virus does not develop any further without its being already completely killed off; death does not supervene before several months have passed, involving the complete inactivation of the virus.

That the deleterious action of the antigenic component of the fixed virus, however, is primarily due to the carbolic acid and not to the temperature of 37° C. follows obviously from the fact, that the chloroformized vaccine, although exposed to this increase of temperature during a much longer period, exceeds the carbolized vaccine by far in activity. Still it may be admitted that the relatively satisfactory results obtained with carbolized vaccines in many Institutes will surely be owing to the rests of fixed virus having survived. But doubtlessly these same traces of live virus must be claimed responsible for the many „treatment accidents" due to carbolized vaccine, however reluctantly this may be admitted.

As main argument against this it is usually brought up that the animal test in its most sensitive form *viz.*, intracerebral inoculation in this case always proves negative.

But Maria J. Otten-van Stockum has rightly pointed out that animal test with 1 or 2 guinea pigs c.q. rabbits such as it is usual, will completely fall short in the detecting of a trace of live fixed virus such as may be present in carbolized vaccine. This comes to the detection of the d.l.m. at its most extreme limit, thus not in the region of 50 % mortality or more, but nearing 0 % and so it is obvious that here not a few animals, left alone a single one will suffice. A systematic examination of 20 1.25 % carbolized vaccines (10 % suspension of 20 different lots of monkey brain), each time 10 guinea-pigs having been inoculated intracerebrally with 10 mg (0.1 ml of a 10 % suspension), offered 3 samples in which virulent virus could still be detected: out of 3 groups of 10 guinea-pigs, 1, 1 and 3 respectively died from infection with fixed virus. An equal number of 1 ‰ formolized vaccines yielded twice a positive result, 1 respectively 2 guinea-pigs being killed. Whereas the lowest mortality in a lot of 10 test animals still amounts to 10 % and this percentage is attained at in no less than 3 out of 5 positive samples, it is obvious that with as few as 10 guinea-pigs the extreme limit d.l.m. certain has not yet been reached. By using at least 100 guinea-pigs and by reinforcing the injecting dose (which in rabbits may be increased up to the tenfold, *viz.*, 0.5 ml of a 20 % suspension = 100 mg) the presence of fixed virus would have been surely detected in many more samples. For further data the reader may be referred to the extensive treatment of this subject in part II of her treatise (p. 145).

Moreover I may point to the fact that 1.5 ‰ formolized vaccine (5 × 24 hours at 37° C.) in more than 100 samples thus in more than 1000 guinea-pigs, has never induced a fixed virus infection.

Even if this would not be deemed sufficient proof of all fixed virus being actually killed, it seems to me even less acceptable that the superiority of this vaccine to the 1 ‰ formolized vaccine (1 × 24 hours at 37° C.) would be due to the presence of more live virus, and it is to this conclusion the assumption of the authors quoted would lead.

Reverting to the chloroformized vaccine the results compiled from all the experimental research known to me appear to issue in a hardly convincing difference in favour of this vaccine and then merely in its commercial shape. When considered more critically the tests may hardly be claimed comparative as neither in preparation nor in administration equal methods were used for both vaccines. So the nature of the fixed virus as well as the age of the samples varied, whereas as a rule the dosage of the chloroformized vaccine has been higher.

Keeping in mind the slight activity of the carbolized vaccine it none the less might be possible that the chloroformized vaccine would yield more satisfactory results and so in some tests it was compared as to its activity with carbolized vaccine. Thus a same virus suspension (33 % monkey brain) has been worked up partly to formolized, partly to chloroformized vaccine. The 1.5 ‰ formolized vaccine after having been heated at 37° C. during 5 days was further kept at 5° C.; the 1 % chloroformized vaccine was kept at 5° C. during a fortnight and in this period according to Kelser's prescription (7) intensively shaken during 5 minutes thrice daily. Some days later an experiment was put up with guinea-pigs and rabbits, 25 per series and 10 animals as checks; further the usual technique was followed (note p. 116).

For safety's sake on the first day of vaccination 10 guinea-pigs were inoculated intracerebrally with the chloroformized vaccine all of them dying on the 9th day from fixed virus infection, proving this not to have been killed. Already during the immunization period it appeared that this virus had kept up its virulence sufficiently also to act lethally when administered: as many as 13 out of 25 rabbits and 6 out of 25 guinea-pigs died in the course of 10—19 and 10—21 days respectively, whereas not a single lethal case supervened in both series with formolized vaccine. The infection with street virus yielded the following result (see Table XI).

Table XI.

Vaccine	rabbit			guinea-pig		
	course			course		
	immunization (treatment accidents)	infection	controls	immunization (treatment accidents)	infection	controls
ormolized	0/25	5/25	10/10	0/25	10/25	9/10
hloroformized	13/25	6/12		6/25	10/19	

The chloroformized vaccine still containing live virus, it might be expected that it would yield better results than the formolized vaccine. However, the opposite appeared to be the case: whereas the formolized vaccine induced a surviving of 80 and 60 %, the chloroformized vaccine yielded round about 50 %. Obviously here the immunity is lower than it would have been under normal conditions owing to the fixed virus disease endured, as has already been described in connection with the immunization experiments carried out with live unattenuated fixed virus (p. 107 and 108). Here again in view of the 100 % mortality of the checks a weakened resistance in the sence of a negative phase was out of the question.

As the chloroformized vaccine in 1 % concentration appeared to be quite insufficient for the killing of the fixed virus Bandoeng in a fortnight, a more systematic investigation of the rabicide action of chloroform seemed indicated. Therefore chloroform in concentration of 1—5 % was added to equal volumes of 33 % suspension of fixed virus, whereupon the 5 samples of vaccine were kept during a fortnight at 5° C. and shaken as mentioned above. Subsequently half of each sample was kept at room temperature and tested at fixed intervals for the presence of fixed virus by means of intracerebral inoculation of guinea-pigs (see Table XII).

Solely in a 5 % concentration chloroform appears to be able to kill all virus at 5° C. within a fortnight; the 4 % concentration needs a further two weeks, the 3 % still one week more and then it lasts full 30 days, thus 2 months since the commencement of the action of chloroform before all traces of virus have disappeared from 1 and 2 % vaccine (and this in a lot of merely 5 guinea-pigs!).

Table XII.

	Chloroformized vaccine (14 days 5° C.)									
tested after . . . days	5° C.				kept at			22° C.		
	1%	2%	3%	4%	5%	1%	2%	3%	4%	5%
14	8/10	5/10	3/10	3/10	0/10	—	—	—	—	—
21 (7)	3/5	4/5	3/5	3/5	0/5	2/5	1/5	2/5	1/5	0/5
28 (14)	4/5	2/5	1/5	0/5	—	1/5	1/5	0/5	0/5	—
35 (21)	2/5	1/5	0/5	—	—	1/5	0/5	0/5	—	—
45 (31)	3/5	1/5	—	—	—	1/5	0/5	—	—	—
55 (41)	2/5	1/5	—	—	—	1/5	0/5	—	—	—
65 (51)	0/5	0/5	—	—	—	0/5	0/5	—	—	—

At room temperature the virus is acted upon more rapidly in all of the concentrations, but nevertheless in the 1 % vaccine merely after an interval of at least 52 days the virus is killed off completely, at least has lost all of its virulence.

Therefore in a further experiment the content of chloroform has been doubled and in order to be on the safe side the period of its action at 5° C. has been extended up to 70 days. Intracerebral inoculation of 10 guinea-pigs did not cause any lethal case. Next to the formolized vaccine which had been prepared simultaneously and further on had been kept at 5° C. a fresh formolized vaccine was prepared, of course in a suspension of equal concentration, as a check on the activity of the former (see Table XIII).

Table XIII.

Vaccine	course		
	immunization (treatment accidents)	infection	controls
2 % chloroformized vaccine (35%) 70 days at 5° C.	0/30	6/30 (20%)	10/10 (100%)
1.5% formolized vaccine (10%) 5 days at 37° C., 65 at 5° C.	0/30	4/30 (13.3%)	
1.5% formolized vaccine (10%) 5 days at 37° C.	0/30	2/30 (6.6%)	

According to the results of this experiments in rabbits which for the rest had been vaccinated and injected in the usual way, the chloroformized vaccine induces a higher rate of mortality than the carbolized vaccine, the more so when compared with the freshly prepared lot. The difference, however, is not very pronounced.

The period of action of the chloroform on a 33 % suspension at low temperature being long, it has finally been ascertained in how far without impairing the antigenic value it might be shortened by means of a lesser concentration of the suspension and exposition at room temperature. When after some preleminary tests a 1.5 % suspension appeared sufficient for a complete killing of a 10 % suspension of the virus at room temperature in a fortnight, a final experiment was put up with a freshly prepared formolized vaccine. The result of this experiment in which no treatment accidents had occurred consisted in 35 % mortality (7/20) among the rabbits treated with chloroformized vaccine and 10 % (2/20) among those treated with formolized vaccine against 100 % (10/10) of the checks.

Although these results do not point to any pronounced superiority of formolized vaccine and for a definite conclusion an investigation on a more extensive scale is needed, all these experiments in question yield results in favour of the formolized vaccine. The total results of these experiments, a mortality of 12 % (9/75) and 31.6 % (19/60) respectively, even yield a difference exceeding the probable deviation (18.6 % ± 4.7).

Moreover it seems to me that even in case of equality in activity and keeping quality for both vaccines, preference should be claimed for the formolized vaccine owing to its shorter period of preparation.

4. INFECTIOSITY AND ANTIGENIC VALUE AFTER CENTRIFUGATING.

In a comparative examination of the results with vaccine employed in antirabic treatment OKUWADA (11) for both live and dead etherized vaccine found that after 15 minutes centrifugating the supernatant liquid acted as strongly as the complete non centrifugated suspension; for live vaccine the infectiosity even appeared to be the same. At first view these results seem contrary to reason and the more so as they are but weakly substantiated by experimental data. Still a closer investigation appeared to be worth while, as even if the nerve tissue as such has to be disclaimed as a cause of „treatment accidents" still ceteris paribus the treatment with a virus low in tissue content remains desirable.

I started from a 10 % fixed virus suspension (a mixture of 3 monkey brains) half of which was centrifugated during 15 minutes at 3000 turns; the supernatant still turbid liquid was drawn off and the sediment filled up to the original volume. Then from the 3 samples series of 10- (c.q. 2- and 5-)fold dilution were made and 0.1 ‰ of each dilution within the limits estimated for 100—0 % mortality (10^{-3}—7^{-3}) inoculated intracerebrally into 5 guinea-pigs. The data from these 5 experiments in which always a same fixed virus has been used are inserted in Table XIV.

Quite contrary to the results of OKUWADA (11) the supernatant liquid merely in the highest concentration (1 : 1000) appears to

Table XIV.

10% — monkey brain fixed virus

Dilution	Intracerebral injection	full suspension						centrifugated											
								sediment						supernatant liquid					
		1	2	3	4	5	total	1	2	3	4	5	totai	1	2	3	4	5	total
10^{-3}	0.1	5/5	—	—	—	—	5/5	5/5	—	—	—	—	5/5	5/5	5/5	5/5	—	—	15/15
10^{-4}	0.01	5/5	—	5/5	5/5	5/5	20/20	5/5	—	5/5	5/5	5/5	20/20	4/5	5/5	5/5	4/5	4/5	22/25
5×10^{-5}	0.005	5/5	5/5	—	—	—	10/10	5/5	5/5	—	—	—	10/10	3/5	5/5	—	—	—	8/10
2.5×10^{-5}	**0.0025**	5/5	5/5	—	5/5	5/5	20/20	5/5	5/5	—	5/5	5/5	20/20	1/5	5/5	—	2/5	2/5	10/20
10^{-5}	0.001	2/5	5/5	5/5	5/5	5/5	22/25	3/5	5/5	5/5	3/5	3/5	19/25	1/5	3/5	1/5	1/5	2/5	8/25
5×10^{-6}	0.0005	—	—	—	4/5	4/5	8/10	—	—	—	3/5	4/5	7/10	—	—	—	1/5	1/5	2/10
10^{-6}	0.0001	—	—	4/5	2/5	3/5	9/15	—	—	2/5	2/5	2/5	6/15	—	—	0/5	1/5	1/5	2/15
5×10^{-7}	0.00005	—	—	—	3/5	2/5	5/10	—	—	—	0/5	1/5	1/10	—	—	—	0/5	0/5	0/10
10^{-7}	0.00001	—	—	—	2/5	1/5	3/10	—	—	—	0/5	0/5	0/10	—	—	—	0/5	0/5	0/10

possess a same infectiosity as the full suspension, deviating increasingly at stronger dilution [1]).

At the level of the d.l.m. certa of the full suspension (which since many years for the monkey brain fixed virus of Bandoeng has been kept constantly at 0.0025 mg) the rate of mortality for the supernatant liquid has already dropped to its half, whilst it reaches zero at a dilution in which the full suspension still induces a mortality of 50 %. As might be expected the concentration of the fixed virus in the sediment is lower than in the original full suspension; it is true that its d.l.m. certa is still the same but then the mortality induced by the sediment lags more and more behind that of the full suspension. The curve in which the completely comparable

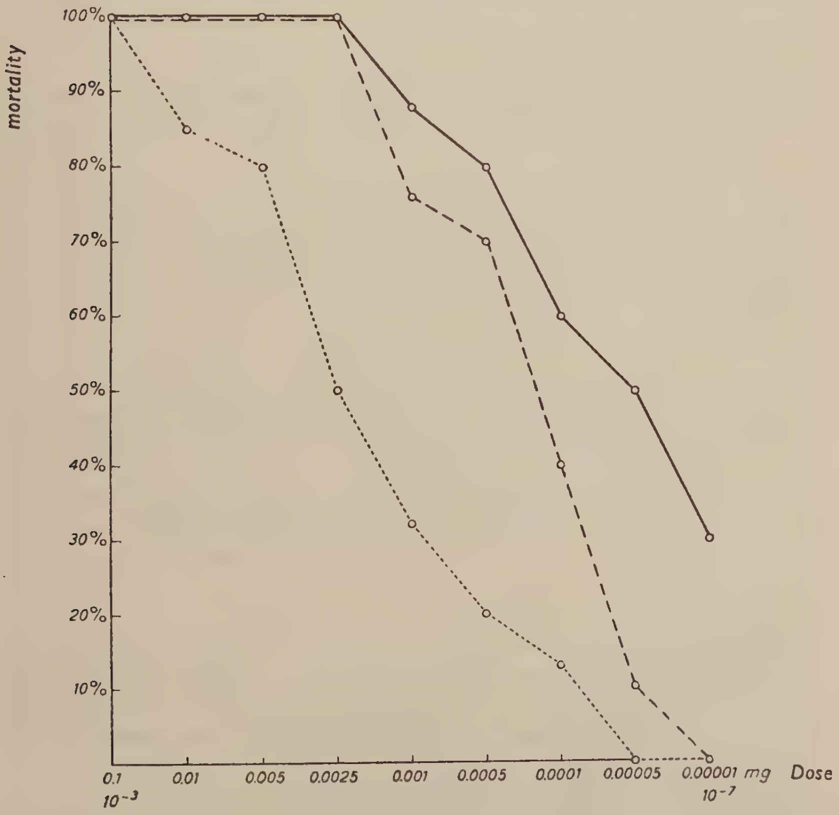


Fig. 1. Intra-cerebral inoculation, virus fixe monkey.
——— 10 % virus fixe suspension.
– – – – centrifugate.
……… supernatant liquid.

---

[1]) Although the supernatant liquid is still rather turbid and thus may be called a suspension, it actually contains much less nerve substance than both other liquids. So for its dosage only the unities of volume not of weight are valid.

rates of mortality for 3 groups of 125 guinea-pigs are recorded, shows more distinctly still the ratio of the concentration of the fixed virus in the 3 suspensions.

The 3 suspensions have besides been tested as to their antigenic activity. Therefore from the 3 suspensions formolized vaccine has been prepared by means of which rabbits, 20 per series were immunized in the usual way; only the dosage of the thinnest suspension (the supernatant liquid) has been 10 times stronger in the 2nd experiment (*4a*). In the first experiment (*3a*) the 3 suspensions have been diluted tenfold, thus into a 1 % suspension of which successively 0.3 (3 mg), 0.7 (7 mg) and 1 ml (10 mg) were injected. In experiment *4a* the same volumes but undiluted were injected. The data are collected in Table XV.

Table XV.

Formolized vaccine	Experiment 3*a*	Experiment 4*a*	total
Full 10%-suspension	1/20 (5 %)	1/20 (5%)	2/40 (5%)
Centrifugate (filled up)	2/20 (10%)	4/20 (20%)	2/40 (15%)
Supernatant liquid	8/20 (40%)	7/20 (35%)	15/40 (37.5%)
Controls	10/10 (100%)	10/10 (100%)	20/20 (100%)

Thus not only the infectiosity but also the antigenic activity of the supernatant liquid lags far behind that of the full suspension, even at a tenfold dosage. Although the total number of animals for each sample of vaccine does not exceed 40, the difference amounts already to more than thrice the standard deviation (32.5 % σ 8.4), the difference when compared with the sediment is already significant, exceeding already the probable deviation (22.5 % ± 6.4).

5. Mode of immunization.

Finally one further experiment has been carried out in order to ascertain whether by means of daily injection another c.q. better immunizing effect might be obtained than by means of inoculation in 3 steps.

In this experiment the dosage for rabbits amounted to 20 mg partly injected as usual in 3 steps (3, 7 and 10 mg) in the course of a fortnight, partly daily in slowly increasing doses (6 × 1, 4 × 1.5 and 4 × 2) covering a same period. The guinea-pigs received 10 mg divided in 2, 3 and 5 mg, respectively 8 × 0.5 and 6 × 1 mg. Moreover a same number of animals has been injected with half the dose, hoping that with thus limiting dose any differences might show clearer. The results of the intramuscular injection following as usual after 30 days have been inserted in Table XVI.

Table XVI.

Formolized vaccine	Rabbit	Guinea-pig
20 mg (3 injections)	0/20 (0%)	—
20 ,, (14 ,,)	1/20 (5%)	—
10 ,, (3 ,,)	6/20 (30%)	1/20 (5%)
10 ,, (14 ,,)	7/20 (35%)	2/20 (10%)
5 ,, (3 ,,)	—	8/20 (40%)
5 ,, (14 ,,)	—	9/20 (45%)
Controls	10/10 (100%)	10/10 (100%)

The formolized vaccine does not show any difference in favour of the daily injection, confirming the (unpublished) results of MARIA J. OTTEN-VAN STOCKUM which neither issued in a clear difference. Also the scarce investigations which can be found in literature, lead to a same result.

My own material discussed already in another connection on p. 105 shows a difference in favour of the daily injection, but the difference was not only slight but the irregular partition of treatment accidents prevented an exact comparision of both groups. This appears from the following figures, the results of 2 experiments with rabbits (5 mg) and guinea-pigs (2 mg).

Rabbit.
After daily injection 10/31 = 32.2 % mortality.
(treatment accidents 9/40 = 22.5 %)
After intermittant injection 11/27 = 40.7 % mortality.
(treatment accidents 13/40 = 32.5 %)

Guinea-pig.
After daily injection 5/38 = 31.1 % mortality.
(treatment accidents 2/40 = 5.0 %)
After intermittant injection 8/34 = 25.5 % mortality.
(treatment accidents 6/40 = 15.0 %)

At daily injection treatment accidents were least and the immunizing effect was slightly better. Apparently with intermittant treatment the level of immunity decreases more strongly, owing to the enduring of a fixed virus infection of a severe character. The only experiment in which no treatment accidents followed (guinea-pig 1 mg) did not show any difference for both modes of injection; the result was 5/15 = 33 % mortality.

The highest subcutaneous vaccinating dosage which does not yet induce fixed virus infection for the rabbit amounts to about 3 mg, for the guinea-pig to 1 mg, but with this limiting dose a by no means maximal immunity is reached. For the rabbit an increase in dosage ceteris paribus results in a marked number of treatment accidents without any increase of the immunizing effect. Only with a daily dosage divided over a longer period (4 weeks) mortality owing to fixed virus and street virus infection drops to ± 10 %, for guinea-pigs even lower. Here, however, the lengthening of the interval between the beginning of the immunization and the infection up to 40 days will doubtlessly have improved the results. It seems very well possible, starting from a very small dose (0.01 mg or less) and dividing the dosage over a longer period of immunization to arise at 100 % surviving without any treatment accidents, but in man a similar method for the attaining of a maximal effect is not practicable.

Therefore it seems strange that the antirabic vaccination of man although widely varying in duration is still administered daily, in the same way as PASTEUR did in the first case in 1886. This keeping to a daily or several times daily injection according to tradition is the more to be wondered at, as in the course of years next to the dry cord method numerous modifications have been accepted, most of which not in need of and but weakly based on experimental results. The question, however, whether a few injections with an interval of 5—7 days might suffice, is of great importance and it seems to me that experimental research in this direction is urgently needed.

Summary.

1. In the above the statistical work of MARIA J. OTTEN-VAN STOCKUM was referred to which has thrown full light on the results of the antirabic treatment with monkey brain fixed virus, both live and dead (formolized vaccine) in man. The distinct shortening of the period of incubation which has been ascertained in cases of rabies treated with the latter vaccine warrants the hope that by these means the immunization process will reach its acme still more rapidly and that therefore within the first 30 days following the starting of the treatment, that is within the ,,failure'' limit now accepted, mortality may be reduced still further.

The results of her experimental work with the fixed virus vaccine which have been shortly summarized, completely confirmed the validity of her method of analysis.

My own experiments led to similar results: MARIA J. OTTEN-VAN STOCKUM using the usual method of immunization (20 mg in 3 steps) after 30 days followed by intramuscular infection with street virus, noted a mortality of 9.6 % (15/155) among her rabbits against 96 % (58/60) among the controls. A still lower rate of mortality was recorded as the average of 13 experiments 9.1 % (26/285) against 99 % (129/130) in the controls.

2. The formolized vaccine, ready for use as a 2 % suspension stored at room temperature, appeared to decrease in activity fairly rapidly. Stored as a 10 % suspension at 5° C. it did practically not decrease in activity, so that decentralization of the treatment with an adequate vaccine may be warranted.

3. The assertion as to the superiority of the chloroformized vaccine to carbolized vaccine has been critically discussed and by means of an analysis of the investigations published thus far it has been demonstrated that at most it may be recognized for a commercially produced chloroformized vaccine.

When the original method of preparation had been followed, chloroformized vaccine in 1 % concentration appeared unable to kill the fixed virus Bandoeng within the period of 14 days at 5° C. as indicated; at least a fifthfold concentration was needed. A few comparative experiments issued in a constant although not very pronounced difference in favour of the formolized vaccine.

4. The testing of a 10 % suspension before and after centrifugating showed that both infectiosity (virulence) and antigenic activity of the supernatant liquid lagged far behind those of the full suspension (and the centrifugate).

5. A single experiment with daily and intermittant (weekly) administration of the immunizing dose did not result in any difference in favour of the daily injection.

Literature.

1. M. F. Barnes, A. N. Metcalfe, W. J. Lentz and W. E. Martindale, J. Am. Vet. Med. Assoc. **76,** 34, 1930; **84,** 740, 1934. – 2. J. Dodéro, Ann. d. l'Inst. Pasteur **61,** 193, 1938. – 3. J. Genevray et J. Dodéro, Ann. d. l'Inst. Pasteur **52,** 352, 1934. – 4. K. Habel, Public Health Reports **55,** 1473, 1619, 1940; **56,** 641, 1941. – 5. H. N. Johnson and C. N. Leach, Am. J. Hygiene **32,** 46, 69, 74, 1940. – 6. H. N. Johnson and C. N. Leach, Am. J. Trop. Med. **20,** 335, 1940. – 7. R. A. Kelser, J. Am. Vet. Med. Assoc. **30,** 595, 1930. – 8. P. Lépine et V. Sautter, C. R. d.l. Soc. Biol. **127,** 192, 1938. – 9. A. G. McKendrick, Bull. Health Org. League of Nations Vol. **1—9,** 1932-40. – 10. R. Nitsch, Centralbl. f. Bakt. I **42,** 647, 775, 1906. – 11. N. Okuwada, Kitasato Arch. Exp. Med. **10,** 279, 1933. – 12. Maria J. Otten-van Stockum, New Principles of Anti-rabic Treatment and Rabies Statistics. Martinus Nijhoff, The Hague, 1935. – 13. Maria J. Otten-van Stockum, Gen. T. Ned. Indië **1941,** 1002. – 14. G. Proca and S. Bobes, Bull. Health Org. League of Nations **9,** 79, 1940. – 15. J. Reichel and J. E. Schneider, J. Am. Vet. Med. Assoc. **84,** 752, 1934. – 16. H. W. Schoening, J. Am. Vet. Med. Assoc. **76,** 25, 1930. – 17. L. T. Webster, J. Exp. Med. **70,** 87, 1939. – 18. L. T. Webster, Am. J. Hygiene **30,** 113, 1939. – 19. L. T. Webster, J. Am. Vet. Med. Assoc. **96,** 65, 1940. – 20. R. G. W. Wyckoff and C. E. Beck, J. Immunology **39,** 17, 1940. – 21. R. G. W. Wyckoff and W. C. Tesar, J. Immunology **40,** 383, 1941.

Bandoeng (Camp for families), May 31, 1944.

(From the „Rijks Instituut voor de Volksgezondheid" at Utrecht).

STUDIES ON STAPHYLOCOCCI

II. ANTISTAPHYLOCOAGULASE IN HUMAN SERUM

by

J. H. BEKKER

(Received April 17, 1947).

Certain *Staphylococcus* strains possess the power to clot plasma, and the ability to produce the clotting agent (staphylocoagulase) is used as a characteristic for the pathogenicity of a *Staphylococcus* strain (1).

As in natural and experimental infections antibodies are produced as a response to different bacterial products, the possibility was suggested that an antibody neutralising staphylocoagulase may be present in the blood. GROSS (2), WALSTON (4) and LOMINSKI and ROBERTS (3) found in human and in rabbit sera a substance capable of inhibiting the clotting of plasma by coagulase; other investigators, however, failed to demonstrate this anticoagulase.

In the course of an investigation into the nature of coagulase it seemed important to ascertain whether the results of these investigations could be confirmed.

Hundred human sera, forwarded to our Institute for the Wassermann reaction were examined for the presence of anticoagulase.

Staphylocoagulase was prepared by growing for 24 hours at 37° C. a *Staphylococcus aureus* strain producing highly active coagulase in broth containing 10% rabbit plasma (0.4% sodium citrate). The partially clotted culture is then shaken until the clot disintegrates; after filtration through paper the culture is passed through a L_3 candle, and the sterility of the filtrate ascertained (the filtrate remains suitable for the tests for several weeks if kept at −3° C.). The minimal coagulating dose (M.C.D.) for a well clotting plasma is determined, *i.e.*, the smallest amount of filtrate which in 24 hours at 37° C. causes a noticeable clot in 10 times with saline diluted rabbit plasma.

The test with the inactivated sera was carried out as follows: to tubes containing 0.5 ml of serial doubling dilutions of serum in saline, and to a control containing 0.5 ml saline, 0.2 ml of coagulase (30 M.C.D.) is added.

After incubating for 90 min. at 37° C. each tube receives 0.3 ml of 3 times with saline diluted rabbit plasma (the same plasma as used for determining the M.C.D.). The final dilution of the plasma is 1 : 10, the sera in such a manner diluted that the final serum dilutions are 1 : 2, 1 : 4, 1 : 8, etc. Readings were made after incubating the tubes for 24 hours at 37° C. and the degree of clotting (designated as —: no clot; + : small floating clot; ++: large clot; +++: solid clot) in each tube noted.

Among the 100 human sera studied an anticoagulase, *viz.*, a substance inhibiting coagulase could often be found. The titre of this substance differs considerably with the various sera (Table I).

Table I.

Occurrence of anticoagulase in the 100 investigated sera.

Number sera	anti-staphylocoagulase titre										
	negative	1 : 2	1 : 4	1 : 8	1 : 16	1 : 32	1 : 64	1 : 128	1 : 256	1 : 512	1 : 1024
100	5	14	18	11	8	7	10	13	2	8	4

Some of the properties of this anticoagulase were studied. Anticoagulase was found to be relatively heat-stable, withstanding 65° C. for 30 min., but being almost completely destroyed after heating for 30 min. at 70° C. (Table II).

Table II.

Influence of inactivating on anticoagulase titre.

Heating-time	Serum T : dilutions										
	1 : 2	1 : 4	1 : 8	1 : 16	1 : 32	1 : 64	1 : 128	1 : 256	1 : 512	1 : 1024	C
0 min. 56° C.	—	—	—	—	—			—	+++	+++	+++
0 min. 65° C.	—	—	—	—	—		—	++	+++	+++	+++
0 min. 70° C.	—	+	+++	+++	+++	+++	+++	+++	+++	+++	+++
0 min. 80° C.	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++

Lominski and Roberts ascertained that whereas anticoagulase was frequently found to be present in human sera only a few human plasmas inhibited the clotting by coagulase. This difference was explained by the fact that coagulase has a greater affinity for fibrinogen than for anticoagulase and the presence of fibrinogen masks the activity of anticoagulase in plasma. Addition of fibrinogen (plasma) has no effect when anticoagulase had been incubated with coagulase (Table III).

Quantitative relations exist between anticoagulase and coagulase; by keeping the fixation-time constant (90 min.) a fixed amount of anticoagulase can neutralise only a fixed amount of coagulase (Table IV). For neutralising a certain amount of coagulase (30 M.C.D.) a minimum fixation-time is required and an increase in fixation time does not increase the rate of inhibition (Table V).

Table III.

Affinity of coagulase for anticoagulase and for fibrinogen.

	Serum T : dilution										
	1 : 2	1 : 4	1 : 8	1 : 16	1 : 32	1 : 64	1 : 128	1 : 256	1 : 512	1 : 1024	C
Serum + coagulase; after 90 min. + plasma:		—	—	—	—	—	—	—	+++	+++	+++
Serum + plasma; after 90 min. + coagulase:	—	—	+	+++	+++	+++	+++	+++	+++	+++	+++
Serum + coagulase + plasma simultaneously:	—	++	+++	+++	+++	+++	+++	+++	+++	+++	+++

Table IV.

Quantitative relations between anticoagulase and coagulase.

Staphylo-coagulase	Serum T : dilutions										
	1 : 2	1 : 4	1 : 8	1 : 16	1 : 32	1 : 64	1 : 128	1 : 256	1 : 512	1 : 1024	C
1 M.C.D.	—	—	—	—	—	—	—	—	—	—	—
5 M.C.D.	—	—	—	—	—	—	—	—	—	+	+
10 M.C.D.	—	—	—	—	—	—	—	—	+	+++	+++
20 M.C.D.	—	—	—	—	—	—	—	—	+++	+++	+++
50 M.C.D.	—	—	—	—	—	—	—	+++	+++	+++	+++
100 M.C.D.	—	—	—	—	—	—	+++	+++	+++	+++	+++
200 M.C.D.	—	—	—	—		+++	+++	+++	+++	+++	+++

Table V.

Influence of fixation-time on anticoagulase titre.

Fixation-time	Serum T : dilutions										
	1 : 2	1 : 4	1 : 8	1 : 16	1 : 32	1 : 64	1 : 128	1 : 256	1 : 512	1 : 1024	C
0 min.	—	+	+++	+++	+++	+++	+++	+++	+++	+++	+++
30 min.	—	—	—	++	+++	+++	+++	+++	+++	+++	+++
60 min.	—	—	—	—	—	++	+++	+++	+++	+++	+++
90 min.	—	—	—	—	—	—	—	—	+++	+++	+++
120 min.	—	—	—	—	—	—	—	—	+++	+++	+++
240 min.	—	—	—	—	—	—	—	—	+++	+++	+++
360 min.	—	—	—	—	—	—	—	—	+++	+++	+++

The fixation of coagulase with anticoagulase appeared to be reversible. Serum T showed in a dilution 1 : 256 no clotting after

24 hours at 37° C. Half of this dilution kept for a further 24 hours at 37° C. remains unclot, whereas addition of diluted plasma to the other half causes clotting after 24 hours at 37° C.

The properties of anticoagulase concur with the generally accepted characteristics of antibodies, and an investigation was made to ascertain whether anticoagulase could also be found in the serumprotein fraction which contains most of the antibodies, *viz.*, the γ globulin fraction.

The sera were therefore fractionated in the usual way with ammonium sulphate into albumin and globulin fractions and the latter subsequently into euglobulin and pseudoglobulin. Tables VI and VII show that anticoagulase was not found in the albumin fraction whereas the globulin fraction contained practically all the inhibiting substance; on further fractionation most of the anticoagulase came down with the pseudoglobulin and less with euglobulin.

Table VI.

Relation of anticoagulase to serum protein fractions (salting-out with ammonium sulphate)

Serum fractions	Serum T: dilutions										
	1 : 2	1 : 4	1 : 8	1 : 16	1 : 32	1 : 64	1 : 128	1 : 256	1 : 512	1 : 1024	C
whole serum	—	—	—	—	—	—	—	—	+++	+++	+++
globulin	—	—	—	—	—	—	—	+++	+++	+++	+++
euglobulin	—	—	—	++	+++	+++	+++	+++	+++	+++	+++
pseudoglobulin	—	—	—	—	—	—	—	+++	+++	+++	+++
albumin	—	+	+++	+++	+++	+++	+++	+++	+++	+++	+++

Table VII.

Relation of anticoagulase to serum protein fractions (salting-out with ammonium sulphate)

Serum fractions	Serum v. d. G.: dilutions										
	1 : 2	1 : 4	1 : 8	1 : 16	1 : 32	1 : 64	1 : 128	1 : 256	1 : 512	1 : 1024	C
whole serum	—	—	—	—	—	—	—	—	—	+++	+++
globulin	—	—	—	—	—		—	—	++	+++	+++
euglobulin	—	—	—	+	++	+++	+++	+++	+++	+++	+++
pseudoglobulin	—	—	—	—	—	—	+	+++	+++	+++	+++
albumin	—	++	+++	+++	+++	+++	+++	+++	+++	+++	+++

The fractionation experiments with sodium sulphate are in good accordance with these findings (Table VIII).

Table VIII.

Relation of anticoagulase to serum protein fractions
(salting-out with sodium sulphate)

Serum fractions	Serum T: dilutions										
	1 : 2	1 : 4	1 : 8	1 : 16	1 : 32	1 : 64	1 : 128	1 : 256	1 : 512	1 : 1024	C
whole serum	—	—	—	—	—	—	—	—	+++	+++	+++
glubulin	—	—	—	—	—	—	—	++	+++	+++	++
euglobulin	—	+	+++	+++	+++	+++	+++	+++	+++	+++	[illegible]
pseudoglobulin	—	—	—	—	—	—	+	+++	+++	+++	+++
albumin	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++

After treatment of the sera with 20% ethanol (a method for obtaining γ globulin) anticoagulase was found (cf. Table IX) mainly in the filtrate (containing but little γ globulin) and less in the sediment (mostly γ globulin).

Table IX.

Relation of anticoagulase to serum protein fractions
(salting-out with ethanol).

fractions	Serum T : dilutions										
	1 : 2	1 : 4	1 : 8	1 : 16	1 : 32	1 : 64	1 : 128	1 : 256	1 : 512	1 : 1024	C
precipitate	—	—	+	+++	+++	+++	+++	+++	+++	+++	[illegible]
filtrate	—	—	—	[illegible]	—	[illegible]	[illegible]	++	+++	+++	[illegible]

These experiments made it probable that anticoagulase was not linked with the γ globulin fraction but with the α or β fraction.

By means of electrophoresis pure γ globulin was obtained from the sera T and v. d. G. As the electrophoretic isolation of α and β globulin is much more difficult and as it was known that albumin did not contain anticoagulase the albuminfraction was intentionally contaminated with α and β globulin. Tables X and XI show in accordance with the salting-out experiments that γ globulin does not (or only to a slight degree) contain anticoagulase but that the inhibiting substance can be found in the α or β globulin fraction of the serum proteins.

Table X.

Relation of anticoagulase to serum protein fractions (electrophoretic isolation)

serum fractions T:	dilutions										
	1 : 2	1 : 4	1 : 8	1 : 16	1 : 32	1 : 64	1 : 128	1 : 256	1 : 512	1 : 1024	C
otal serum protein	n.c.1)	—	—	—	—	—	—	—	+++	+++	+++
-globulin	n.c.	n.c.	(1 : 12) +++	+++	+++	+++	+++	+++	+++	+++	+++
lbumin α and β globulin	n.c.	(1 : 5) —	—	—	—	++	+++	+++	+++	+++	+++

1) n.c. = not carried out.

Table XI.

Relation of anticoagulase to serum protein fractions (electrophoretic isolation)

serum fractions v. d. G.	dilutions										
	1 : 2	1 : 4	1 : 8	1 : 16	1 : 32	1 : 64	1 : 128	1 : 256	1 : 512	1 : 1024	C
otal serum protein	n.c.1)	—	—	—	—	—	—	—	+	+++	+++
-globulin	n.c.	n.c.	(1 : 13) +++	+++	+++	+++	+++	+++	+++	+++	+++
lbumin, α and β globulin	n.c.	—	—	—	—	—	+	+++	+++	+++	+++

1) n.c. = not carried out.

The protein composition of serum T and serum v. d. G. is given below:

	serum T	serum v.d.G.	normal
albumin	45.3%	55.2%	60%
α globulin	9.5%	5.8%	6%
β globulin	15.3%	11.7%	13%
γ globulin	29.9%	27.3%	21%

In both sera a slight increase in the γ globulin is found. Serum T showed a striking deviation of the β globulin pattern, especially in the descending boundary. It has however not been ascertained as yet whether any relation exists between this phenomenon and anticoagulase.

The experiments in the present study confirm the statements of Gross, Walston and Lominski and Roberts that in human serum a substance can be found capable of inhibiting the clotting of plasma by staphylocoagulase. This anticoagulase shows antibody characteristics, it is however probably not linked with the γ globulin but with the α or β globulin fraction. Experimental proof

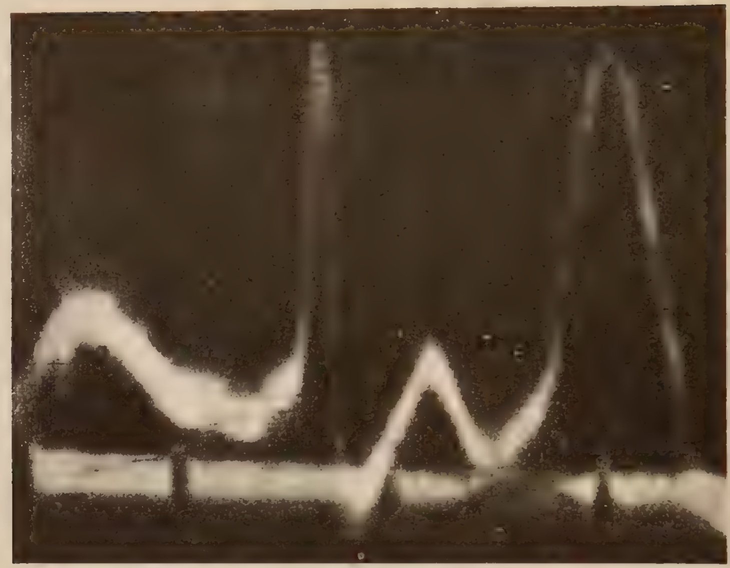


Fig. 1. Electrophoretic pattern of serum T, descending boundary.

that this anticoagulase is a real antibody, *viz.*, that it is formed as a response to the antigen (coagulase) has not been furnished as yet.

Summary.

A substance inhibiting the coagulation of plasma by staphylocoagulase can be found in human sera; this substance has antibody characteristics and is present in the α or β globulin.

I am greatly indebted to Dr. L. W. JANSSEN (State Institute for Veterinary Research, Amsterdam) for carrying out the electrophoretic analyses, and to Miss Dr. L. H. C. VAN DE GRAAF (Municipal Hospital, Zaandam) for supplying sera.

References.

1. J. H. BEKKER, Antonie van Leeuwenhoek **13**, 63, 1947; Geneeskundige Gids **25**, 199, 1947. — 2. H. GROSZ, Klin. Wschr. **12**, 304, 1933. — 3. I. LOMINSKI and G. B. S. ROBERTS, J. Path. Bact. **58**, 187, 1946. — 4. H. D. WALSTON, J. Hyg. **35**, 549, 1935.

(From the Biophysical Research Group Utrecht—Delft under the direction of A. J. KLUYVER and J. M. W. MILATZ).

SOME OBSERVATIONS ON THE CULTURE, PHYSIOLOGY AND MORPHOLOGY OF SOME BROWN-RED *RHODOSPIRILLUM*-SPECIES

by

G. GIESBERGER

(Received June 10, 1947).

1. INTRODUCTION.

During an investigation on the genus *Spirillum* carried out by the author (4) 10 years ago in the Laboratory for Microbiology at Delft incidentally also some observations on spirilla were made, which differed from the heterotrophic colourless species by their capacity to carry on a photosynthetical mode of life. Next to various strains which could be identified as *Rhodospirillum rubrum*, also some strains were isolated from mud of a ditch, which clearly distinguished themselves from these, because they did not contain a purple-red, but a definitely brown-red pigment-complex. Moreover these strains were obligately anaerobic. A further investigation showed that the isolated strains represented two different species judged by morphological characteristics. Also some preliminary observations on the physiology of these organisms were made which proved that for the photosynthetical CO_2-reduction substances such as acetate, butyrate, lactate, malate, citrate and pyruvate could function as H-donor.

Though at that time opportunity for a more thorough investigation of these organisms was lacking, and a publication of the obtained data did not seem justified as yet, the results still induced KLUYVER and VAN NIEL (7) to include these spirilla in the natural system of classification of bacteria in a newly created genus *Phaeospirillum*. Unfortunately before long the isolated spirilla were lost, so that no further investigation resulted at that time.

Since then VAN NIEL (14) has published a most valuable study on the culture, physiology and morphology of the non-sulfur purple and brown bacteria, in which he describes the physiological and morphological characteristics of a brown-red, photosynthetically active *Spirillum* isolated by him. Although this organism clearly distinguished itself from *Rhodospirillum rubrum* by its brown-red colour, VAN NIEL's conclusion based on a further study of the physiology of the organism was, that the differences in physiological activity were not sufficient to warrant the placing of this organism in a separate genus *Phaeospirillum*. In consequence hereof *Phaeospirillum* Kluyver et van Niel was cancelled by van Niel, and the new spirillum was described under the name of *Rhodospirillum fulvum*.

Rhodospirillum fulvum particularly distinguished itself from *Rhodospirillum rubrum* by its brown-red colour. In accordance herewith van Niel found for *Rhodosp. fulvum* an absorption maximum at 530 μ against 553 μ for *Rhodosp. rubrum*. An obvious distinction is moreover that *Rhodosp. fulvum* is a strict anaerobe, whereas *Rhodosp. rubrum* can effect a normal respiration. Fatty acids and the four carbon dicarboxylic acids, besides ethanol, glucose and aspartic acid were found to be suitable substrates for *Rhodosp. fulvum*. There was no development with thiosulfate.

Van Niel's investigation was seriously hampered by the great sensitiveness of *Rhodosp. fulvum* towards oxygen. An extensive investigation was not possible, as the four isolated strains soon perished.

The data described in this publication on some brown-red spirilla are the result of an investigation carried out in 1946 in the Physical Laboratory at Utrecht, as part of the program of the Biophysical Research Group Utrecht-Delft.

2. Enrichment and isolation.

The brown-red spirilla isolated by me 10 years ago originated from an enrichment culture for purple bacteria according to Molisch (11), which was inoculated with some mud from a ditch, hay being used as an organic substrate.

Rhodospirillum fulvum was isolated by van Niel from an enrichment culture in a glass stoppered bottle (inoculated with a little mud or surface water), which next to the usual mineral salts as $MgCl_2$, K_2HPO_4 and $(NH_4)_2SO_4$ contained $NaHCO_3$ or Na_2CO_3 (as a supply of CO_2) with the addition of caprylate or pelargonate as the only organic substance. However, van Niel mentions that these enrichment cultures only incidentally led to brown-red spirilla, and he emphasizes that a specific enrichment medium for *Rhodosp. fulvum* is not known as yet.

In my attemps to isolate again brown-red coloured, photosynthetically active spirilla, several times enrichment cultures were incubated in the light cabinet at 25° C. In these experiments the above mentioned basal medium (pH ± 7.3) was used, after a single organic substance had been added, for which a number of individual members of the groups of simple alcohols, polyalcohols, fatty acids, hydroxyacids and dibasic acids were used. Though in fact in these enrichment cultures incidentally brown-red spirilla developed and could be isolated (among others with lactate, malate, butyrate, ethanol), nevertheless the results were far from satisfactory. A really good enrichment culture could never be obtained, the results being far too erratic, my results being thus in accordance with those of van Niel. Usually *Rhodosp. rubrum* and other members of the non-sulfur purple or brown rod-shaped bacteria predominate. The exclusive use of the synthetic enrichment media most likely is the reason why van Niel could merely isolate a single brown-red *Rhodospirillum*-species although several are rather common in ditch- and canalwater.

Anyhow during the subsequent investigation it appeared more or less to my surprise that — just as with the heterotrophic *Spirillum* species — a very good and constant enrichment of brown-red spirilla can be secured by means of hay infusion. If namely a glass stoppered bottle filled with water is provided with some hay and the bottle subsequently is inoculated with some mud from a ditch, it is often found that after about one week incubation in the light cabinet at 25° C. the contents of the bottle turn intensely brown-red owing to an abundant development of beautiful spirilla. Among these spirilla mostly two types predominate, namely a very large stout spirillum (under these conditions usually with only $\frac{1}{2}$ to one turn), and a small, slender spirillum. Both types had already been noted by me 10 years ago and subsequently isolated.

The spirilla show a very strong negative aerotaxis. After lifting the stopper of the bottle, the spirilla can be observed to disappear rapidly from the upper layers of the liquid and to aggregate at the bottom of the bottle. This behaviour already suggests the strongly anaerobic nature of these organisms which lateron could be demonstrated on the pure cultures obtained. In view of the anaerobic nature of these spirilla I was somewhat surprised that enrichment cultures of these bacteria could also, and rather often, be obtained from superficially skimmed off canal water.

Another remarkable feature of these enrichment cultures with hay infusion is that not seldom after some weeks tiny green bacteria develop, as a result of which the contents of the bottle turn a dark green. Pure cultures of the bacteria could be obtained via shake-cultures in tap water agar (2 %) provided with 0.05 % K_2HPO_4, 0.05 % $MgSO_4$, 0.1 % NH_4Cl, 0.1 % $Na_2S . 9H_2O$ and 0.1 % $NaHCO_3$, pH = 7.3. They proved to be irregularly shaped bacteria and could be identified with *Chlorobium limicola*.

In connection with the isolation of the spirilla here follows an expedient method for the separating of the spirilla from an enrichment culture, in which always a rather high percentage of „contaminating" bacteria occurs. In this method use is made of the capacity of the latter to move very quickly over comparatively large distances. This principle was for the first time applied by me in my study on the heterotrophic spirilla when attempting to isolate the spirilla occurring in liquid media. For that purpose I let the spirilla present in an enrichment culture swim through a „Pasteur-pipette", which was filled with a sterile liquid medium. A number of the very fast moving spirilla very soon leave the non-motile, resp. less motile, bacteria of the enrichment culture far behind, a fact which can be followed fairly easily under the microscope. By cutting the pipette in the right place it thus was possible to separate a number of spirilla from the ,,contaminating'' bacteria.

The same method has later been applied by MYERS (12) for the mechanical separation of spirilla from crude cultures. The method, however, has now been further simplified by me in the following way.

A tiny drop of the enrichment culture is brought on a glass slide, after which a drop of sterile tapwater or of a nutrient liquid (which in the case of the brown-red spirilla should be boiled beforehand in view of the strongly negative aerotaxis!) is placed on the slide very close to the drop of the enrichment culture. Both drops are now carefully put into contact with each other, and in that way are united into one big, preferably somewhat protracted drop. Through the microscope it can now be observed that the very motile spirilla rapidly disperse into the sterile part of the drop. After some minutes already quite a lot of the spirilla have reached the other extremity of the ,,sterile'' part of the drop. By means of a finely drawn-out micropipette it is possible to remove under the microscope a little of the ,,sterile'' liquid containing a large quantity of spirilla.

Thus very quickly and without much difficulty a particularly effective separation of the spirilla from the non-motile, resp. less motile, organisms in the enrichment culture is secured. Not seldom in using this procedure 80--90 % of the developing colonies after inoculation in a nutrient agar proved to be colonies of the desired spirillum. This, of course, considerably simplifies the necessary microscopical examination of the colonies and the isolation of the spirilla. Naturally the method is not only valuable for the mechanical enrichment of spirilla but can be used whereever the separation of motile from less motile, resp. non-motile, microorganisms is required.

For the isolation of the brown-red spirilla shake-cultures in culture tubes were made [1]. As agar medium peptonagar (Pepton Poulenc) proved to be satisfactory. The pH should be neutral to slightly alkaline. After a lapse of about one week one observes in the agar, between cultures of the much faster developing ,,contaminating'' bacteria, small brown-red coloured colonies which at microscopical examination turn out to consist of spirilla.

To transfer these colonies for further purification, for convenience sake [2], a simple micromanipulator was used by means of which the spirilla were sucked in finely drawn- out micropipettes. For that purpose 1—2 mm thick slices of the agar column were cut with a razor blade. In case there are many colonies of other bacteria in the slices there is a chance that during the cutting of the agar the flat of the cut will become contaminated with these bacteria, so that on sucking the spirilla in the micropipette numerous ,,contaminating'' bacteria will be carried along with the spirilla. However, this can for the greater part be prevented in a simple way by carefully washing the agar slice lying on the slide for some time under a thin jet of tapwater. In this way the flat of the cut can be cleaned to a large extent of loose bacteria. The excess tapwater can then be sucked off with filter-paper, and subsequently the slice of agar superficially dried at 30° C. Thus it can be effected that already after one transfer nearly all ,,contaminating'' bacteria are eliminated.

---

[1] VAN NIEL recommended as a practical measure for isolation purposes (shake-cultures) the use of soft glass rather than of Pyrex tubes, because when the agar has to be removed the former tubes can be easily cut at the bottom. However, the agar column can also rather easily be removed from the glass tube without the need of cutting the tube. Hereto a vigorous air current is blown through a Pasteur-pipette into the tube between the agar column and the wall of the tube. If the air current is strong enough, the air penetrates between the agar wall to the bottom of the tube, finally causing the whole agar column to be pressed out of the tube.

[2] A great advantage of the use of the micromanipulator is the con-

When the agar column does not contain many colonies it is still better to break the agar column cautiously by bending it. Then with a razor blade a slice of the agar can be cut, taking care in doing so that the break remains untouched.

When making transfers of the colonies it is necessary to take into account the strictly anaerobic nature of the brown-red spirilla. Without taking special precautions disappointments are bound to occur because the transferred spirilla will not develop in the fresh agar. These precautions do not merely consist in the boiling of the agar before the transfer in order to remove the oxygen, but als in adding an amount of about 0.01 % $Na_2S \cdot 9H_2O$, and in covering the agar colum with a thin layer of sterile paraffin of low melting point. For safety's sake I also added 0.1 % $NaHCO_3$ to the agar as a CO_2 source though this is not strictly necessary, because the brown-red spirilla can also grow in pepton agar without special addition of CO_2. Lastly the oxygen in the air column above the agar was removed by bringing in the tube some cotton-wool soaked in a mixture of pyrogallic acid and potassium hydroxide and by subsequently closing the tube with a rubber plug. Even with these precautions it is advisable, when making transfers, to blow the spirilla out of the micropipette into the lower part of the liquid agar medium, because the spirilla often refuse to grow in the uppermost agar layer of about 1 cm.

In the course of the investigation about 20 strains of brown-red spirilla were isolated in the described way. The pure cultures were kept at room temperature in day-light as stable cultures in pepton agar. The oxygen was removed from the culture tubes in the above mentioned way. Addition of Na_2S and Na_2CO_3 is not necessary, but covering of the agar with paraffin remains desirable,

---

siderable gain of time obtained. In that way it is often possible to make transfers already after 1—2 weeks. In order to obtain colonies sufficiently big for transferring by hand, one has to wait much longer. Moreover, the use of the micromanipulator offers better chances of avoiding contaminating bacteria.

whilst the agar should be boiled well before transferring. When making transfers it is certainly to be recommended for a successful start of the culture to inoculate heavily, which can be done better by means of a Pasteur-pipette than with a platinum needle.

3. CLASSIFICATION AND IDENTIFICATION OF THE ISOLATED SPIRILLA.

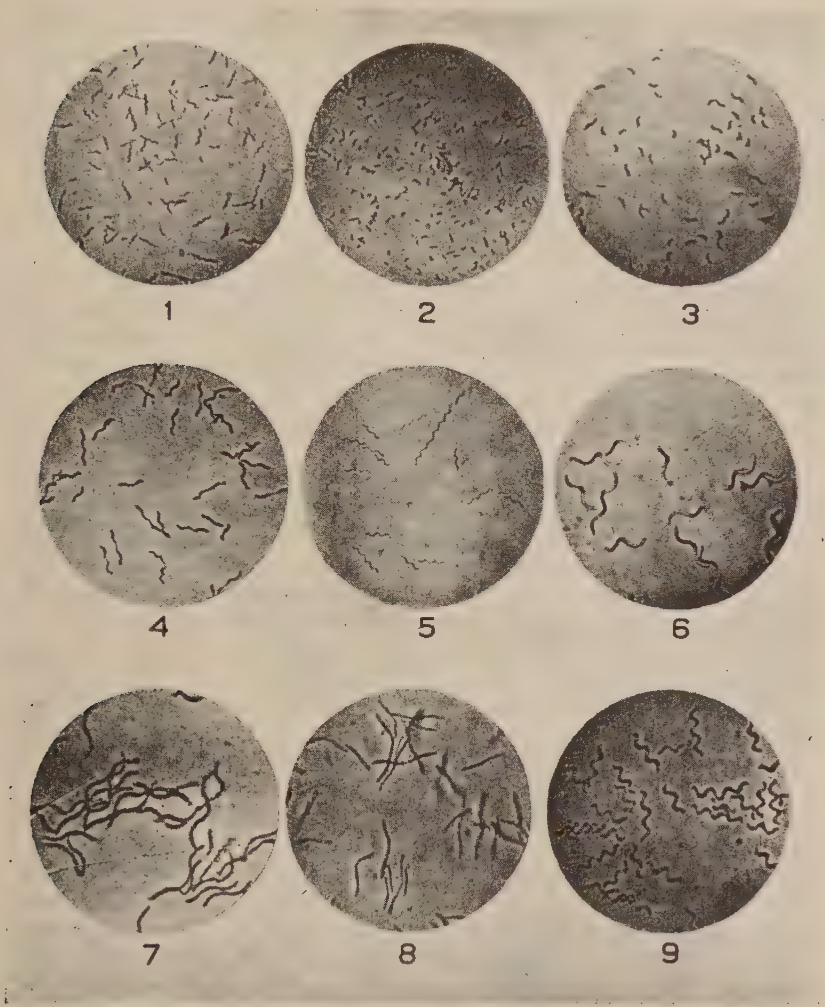
a. Introductory. In accordance with the experiences obtained in my study of the heterotrophic spirilla, VAN NIEL emphatically points out the great morphological variation which *Rhodosp. rubrum* and *Rhodosp. fulvum* show under various environmental conditions. Hereby a good description and identification of these organisms was seriously hampered. Hence VAN NIEL's statement that an adequate description of a species which shows such a pronounced morphological variability must include the characteristics of the organisms as it develops under a variety of environmental conditions (e.g. the reaction of the medium, the nature and the concentration of the nutrient materials present etc.). The judgment of the conditions which should be regarded as representing a more or less normal and significant state is hereby, at least partly, a matter of personal appreciation.

The same difficulty had to be faced when trying to give a morphological characterisation of the brown-red spirilla isolated by me. Although the isolated strains could be separated into three, morphologically clearly distinct groups, the strains comprised in these groups to a large extent exhibit the phenomenon of morphological variation under various environmental conditions (compare fig. 1—9). Even variations in environmental conditions so slight that at the present time it remains impossible to evaluate them, cause one and the same organism to manifest itself either as a nearly straight rod-like bacterium with only faintly developed turns, or in the form of beautifully spiral-shaped cells. Between these extremes various intermediary forms can occur. It is almost impossible to include these divergent forms in a satisfactory morphological characterisation.

With regard to the foregoing remarks it must immediately be emphasized, however, that the spirilla from prolific cultures — with practically all the cells strongly motile — always exhibit normal spiral-shaped cells. The very flatly wound, almost straight cells are only found in cultures which obviously do not represent optimal conditions. An indication hereof is the fact that the cells mostly are but slowly motile, whereas in many cells there is a tendency to retarded division and thus to grow out into very long cells.

However divergent the shape of the turns may be, generally the thickness of the cells does not vary much. In my opinion therefore this quality is one of the most important criteria for the morphological characterisation.

In the description of the morphological properties following below I have attempted to give a characterisation of the cell form of the spirilla in actively proliferating cultures.



Figs. 1—2. *Rhodospirillum fulvum* (Group I). × 460. Cultures of strain 1 in basal medium with lactate and asparagin respectively.
Figs. 3—5. *Rhodospirillum Molischianum* (Group II). Figs. 3 and 4: × 460; Fig. 5: × 345. Cultures of strain 5 in basal medium with ethanol and citrate respectively, and in 0.2 % pepton 0.5 % Ca-lactate.
Figs. 6—9. *Rhodospirillum photometricum* (Group III). Fig. 6, 7, 8: × 460; Fig. 9: ×.345. Cultures of strain 17 in hay-infusion, pepton water, 1 % pepton, 0.5 % Ca-lactate and 0.2 % pepton, 0.5 % Ca-lactate respectively.

b. Morphological properties of the isolated strains.

As mentioned before, the isolated strains fell apart in three clearly distinct groups.

Group I. To this group belong 2 strains[1] (fig. 1—2). Characteristic for these spirilla is the size of the cells which is so small that often it is difficult to get a good idea of their shape. The thickness of the cells is 0.3—0.4 μ, whilst the length is mostly only 2—3 μ. In media to which certain salts of organic acids, for instance malate, have been added, sometimes individual cells can be encountered, which are 4—5 μ long. In case salts of organic acids have been added the spirals often manifest themselves more distinctly, but, in view of the small dimensions of the cells, the width of the spirals is difficult to appreciate.

Group II. To this group belong 8 of the isolated strains (fig. 3—5). To some extent the spirilla in their cell form and size show a certain likeness to *Rhodospirillum rubrum*. The length of the cells usually amounts to 5—10 μ. The thickness of the cells is 0.7—0.9 μ. Generally the cells shows 1—2 complete turns, the length of a turn being 4—6 μ, and the width 1.3—2 μ. As reserve substance volutin is found.

Group III. In this group the remaining 11 strains could be included (fig. 6—9). Characteristic for these spirilla are the large, stout cells. The length of the cells mostly amounts to 14—30 μ, while the thickness of the cells is 1.2—1.5 μ. Generally the cells have 1—2 complete turns. Length of the turn 7—9 μ, width of the turn 4—6 μ. As reserve substance volutin is found.

c. Colour of the isolated strains.

The colour of the colonies and of the stab cultures of all the isolated strains was brown-red, the colour of Group I, however, showing a somewhat redder tinge than that of the other strains[2]. The colour of liquid cultures could often be designated as brown-red to brown-orange. Formation of water soluble, diffusable pigments was not observed.

The absorption spectrum of thin suspensions of living cells in water was determined by the method usually applied for this purpose in the investigations of the Biophysical Research Group (compare Wassink, Vermeulen, Reman and Katz (17)). The apparatus mainly consists of a double monochromator according to van Cittert and an alternating current amplifier according to Milatz (9, 10). For comparison the absorption spectrum of a suspension of living cells of *Rhodospirillum rubrum* is also included in fig. 10.

The figure immediately strikes by the great conformity in the absorption spectra of the three groups of brown-red spirilla. Meanwhile there are also differences. Characteristic for Group III is for

---

[1] Unfortunately both strains died during a severe frost period when the laboratory was not heated.

[2] It is striking that there exist different species of brown-red spirilla, whereas up to now *Rhodospirillum*-species with the same pigment complex as *Rhodosp. rubrum*, but differing from this species in other respects have not as yet been isolated with certainty.

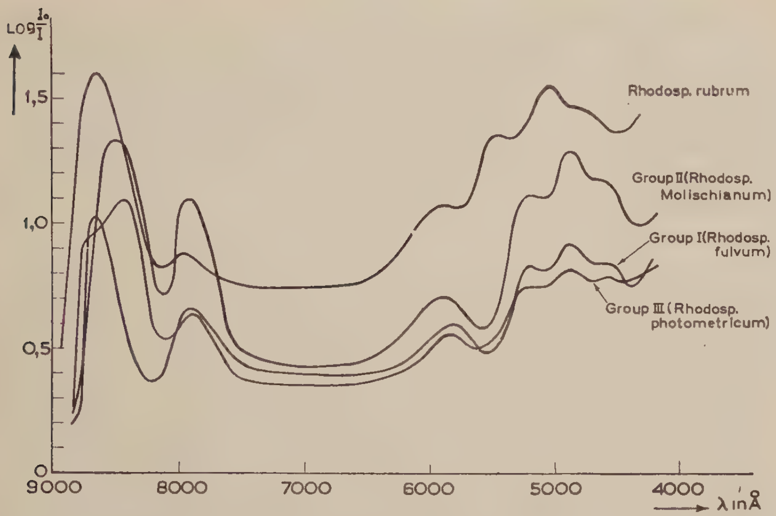


Fig. 10. Absorption spectra of suspensions of living cells of four *Rhodospirillum*-species.

instance the typical slanting maximum (of bacteriochlorophyll) at ± 8500 Å. This can be explained by the presence of two closely adjoining maxima, which in view of the theoretical considerations of WASSINK, KATZ and DORRESTEIN (16) and of FRENCH (3) must be connected with the properties of the proteins, to which the bacteriochlorophyll is bound.

On comparing the spectra with the infrared absorption spectra of various purple sulfur bacteria given by WASSINK *et al.* (16) it is of interest to note that such a flat maximum of the bacteriochlorophyll in the infrared part of the spectrum is encountered in morphologically very divergent groups of organisms. The absorption spectrum of the brown-red spirilla of Group III for instance is in this respect entirely comparable with the absorption spectrum of *Phaeomonas* strain 4 (compare WASSINK *et al.*).

The most typical difference between the absorption spectra of the brown-red spirilla and that of *Rhodosp. rubrum* is the absence of the maximum at 5500 Å in the absorption spectra of the brown-red spirilla. For the rest some more differences in the position of the maxima between the absorption spectra of the brown-red spirilla and *Rhodosp. rubrum* appear at the shorter wave lengths. Moreover, one is struck by the phenomenon, that the maximum at 7900 Å of the bacteriochlorophyll, which manifests itself only faintly with *Rhodosp. rubrum*, is very conspicuous in the absorption spectra of the brown-red spirilla.

From the investigation of MANTEN (8) it is evident, that the maximum at 5500 Å in the absorption spectrum of *Rhodosp. rubrum* is due to the occurrence of the carotenoid spirilloxanthin (rhodoviolascein). From the absorption spectra of the brown-red spirilla thus the conclusion can be drawn that this carotenoid is absent in these organisms. The results of a chromatographical analysis of the pigment complex of one of the strains of Group III were in perfect agreement with this conclusion. By this chromatographical analysis next to bacteriochlorophyll the occurrence of 5 different carotenoids could be established. A detailed report of this investigation will be published elsewhere.

d. Physiological properties of the isolated strains.

For their development the brown-red spirilla are bound to a neutral to slightly alkaline reaction of the medium. The influence of the temperature has not been investigated in detail, but growth of the spirilla was good between 25 and 35° C.

All isolated strains were strictly anaerobic. Further details about the great sensitiveness of these organisms to oxygen have been given in the discussion of the isolation of the spirilla. Adaptation to oxygen has not been observed. In this respect the brown-red spirilla distinguish themselves markedly from *Rhodosp. rubrum* for their growth is strictly dependent on photosynthetical activity, whereas *Rhodosp. rubrum* can also grow in the dark by means of a respiration process.

Gelatin is not liquefied by any of the isolated strains.

e. Biochemical properties of the isolated strains.

In order to ascertain which organic substances could function as a substrate (H-donor) for the development of the spirilla, the isolated strains were inoculated in liquid media to which besides the usual inorganic nutrients and 0.1 % $NaHCO_3$ 1 % of certain organic substances was added.

No differences in this respect could be established between the three groups of strains. All the strains developed more or less satisfactorily with substances such as: acetate, butyrate, malate, lactate, citrate, succinate, ethanol and asparagin. With glucose no growth could be observed of the strains belonging to group II or III[1]).

Thiosulfate nor Na_2S were utilized. The isolated brown-red spirilla thus belong to the typical *Athiorhodaceae*.

f. Identification of the isolated strains.

Besides the well-known and frequently investigated *Rhodosp. fulvum* described by van Niel a few other *Rhodospirillum*-species have been recorded in the literature. So Molisch (11) in his well-known monograph on the purple bacteria has described two *Rhodospirillum*-species, nam ly *Rhodosp. photometricum* and *Rhodosp.*

---

[1]) The strains of group I have not been examined in this respect, because they had already perished at that time.

giganteum, from which only the first species has been studied in pure culture [1]).

Both species have been declared by VAN NIEL to be synonymous with *Rhodosp. rubrum* because in his opinion the morphological properties of the species described by MOLISCH, in view of the great morphological variation of these organisms do not justify a differentiation from *Rhodosp. rubrum*. However, in my opinion this cannot be accepted without further argumentation as MOLISCH explicitly mentions that he possessed an authentic culture of *Rhodosp. rubrum*, and that it thus should be accepted, that MOLISCH for good reasons considered *Rhodosp. rubrum* and *Rhodosp. photometricum* to be different species. Though in fact *Rhodosp. rubrum* shows a great morphological variation, it is still an exception that the individual cells attain a thickness of more than 1 μ (thickness of the cells generally 0.7 μ), whereas for *Rhodosp. photometricum* the thickness of the cells is stated by MOLISCH as 1.4 μ.

In this respect it is also important to mention that VAN NIEL's own comment on the spectroscopic investigation of MOLISCH was, that MOLISCH has actually investigated spirilla with a pigment complex different from that of *Rhodosp. rubrum*! It seems very probable that *Rhodosp. rubrum* and *Rhodosp. photometricum* are two different organisms.

Another question is whether it is justified to maintain a separate species *Rhodosp. giganteum* apart from *Rhodosp. photometricum*. MOLISCH has described the species *Rhodosp. giganteum* on account of the dimensions of this organism in an enrichment culture. A pure culture of the spirillum was not obtained. The differentiation in dimensions between *Rhodosp. giganteum* and *Rhodosp. photometricum*, however, is only slight, and might entirely be attributed to morphological variation. I too have several times observed, in enrichment cultures, a luxurious development of brown-red spirilla having a somewhat more slender appearance than the spirilla belonging to group III generally show in a crude culture. Nevertheless on isolation it always turned out that the developing colonies only consisted of spirilla representing the normal type of group III, so that the primarily observed differences may well be due to morphological variation. This may explain why MOLISCH could not secure pure cultures of *Rhodosp. giganteum*. In my opinion therefore, *Rhodosp. giganteum* should be considered as synonymous with *Rhodosp. photometricum*.

Next to the above mentioned species HAMA (5, 6) has described *Rhodosp. longum* and *Rhodosp. gracile*, though both only on the basis of the shape of the cells in crude cultures. These species with regard to their morphological properties take a somewhat intermediate position between *Rhodosp. rubrum* and *Rhodosp. photometricum*. Since further data about the properties of these species are lacking, the available data on the morphological properties, in view of the great morphological variability, do not justify a differentiation of these species from *Rhodosp. rubrum* resp. *Rhodosp. photometricum*. In accordance with VAN NIEL who considers both species as synonymous with *Rhodosp. rubrum*, in my opinion the species described by HAMA cannot be maintained as separate species.

---

[1]) NAKAMURA (13) in a publication has claimed the isolation of *Rhodosp. giganteum*. However, NAKAMURA's data on the morphological properties of the isolated spirillum are entirely inadequate for this conclusion. As the spirillum isolated by NAKAMURA could utilize Na_2S and thiosulfate as only H-donor, it belongs to the *Thiorhodaceae*.

After this critical review of the *Rhodospirillum*-species recorded in the literature, it remained to ascertain whether the strains isolated by me could be identified with already described species.

Group I. The fact that the dimensions of the cells of both strains isolated by me are identical with those of *Rhodosp. fulvum* has removed any doubt for me that both these strains can be identified with *Rhodosp. fulvum* van Niel.

Group II. With regard to the dimensions of the cells, the strains belonging to group II show a rather close agreement to *Rhodosp. rubrum*, but they differ from these species by the nature of their pigments and by their different behaviour towards oxygen. Obviously here we are dealing with a new, not yet described species. With regard to the fact that MOLISCH was the first to observe brown-red spirilla (see later), the name of *Rhodospirillum Molischianum* is proposed for the organism.

For the sake of completeness the diagnosis of *Rhodospirillum Molischianum* nov. spec. is given here:

Moderately large, very motile, spiral shaped cells. Length of the cells 5—10 μ, thickness of the cells 0.7—0.9 μ. Cells mostly with 1—2 complete turns. Length of the turn 4—6 μ, width of the turn 1.3—2 μ. As reserve substance volutin is found. Obligately photoheterotrophic. As substrate various organic substances, such as acetate, butyrate, malate, lactate, citrate, succinate, ethanol, asparagin can be utilized. Glucose and glycerol are not utilized, no more than Na_2S and thiosulfate. Gelatin is not liquefied. Colour of the cells brown-red. Besides bacteriochlorophyll various carotenoids are found as pigments, but not spirilloxanthin.

Group III. The spirilla belonging to this group are particularly characterized by the stoutness of their cells, the dimension of which are on the whole in good agreement with those given by MOLISCH for *Rhodosp. photometricum*. The photographs of this organism made by MOLISCH on the whole are in good agreement with the appearance of the strains isolated by me.

It is also important to observe that both MOLISCH and I succesfully used the same enrichment medium, namely hay-infusion. It is highly probable, that MOLISCH in doing so has regularly obtained cultures of brown-red spirilla. In accordance with this MOLISCH writes about the enrichment cultures of *Rhodosp. giganteum*, that „das Wasser bis hinauf rotbraun gefärbt erscheint". VAN NIEL also gives his opinion that in view of the spectroscopic investigations of MOLISCH on the pigments of *Rhodospirillum*-species one must conclude that Molisch has investigated spirilla with a brown-red pigment complex.

On the basis of the preceding considerations I conclude that it is justified to identify the strains belonging to group III with *Rhodospirillum photometricum.*

The following diagnosis of *Rhodospirillum photometricum* Molisch can be given:

Large, stout, very motile spiral shaped cells. Length of the cells 14—30 μ, thickness of the cells 1.2—1.5 μ. Cells mostly with 1—2 complete turns. Length of the turn 7—9.5 μ, width of the turns 4—6 μ. Volutin is found as reserve substance. Obligately photoheterotrophic. As substrate various organic substances such as acetate, butyrate, malate, lactate, citrate, succinate, ethanol, asparagin can be utilized. Not utilized are glucose and glycerol, no more than Na_2S and thiosulfate. Gelatin is not liquefied. Colour of the cells brown-red. Besides bacteriochlorophyll various carotenoids occur as pigments, but not spirilloxanthin.

Summary.

From ditch- and canalwater 21 strains of brown-red, photosynthetically active spirilla were isolated. These spirilla distinguished themselves from *Rhodospirillum rubrum* by the composition of their pigment complex and by their behaviour towards oxygen (strictly anaerobic). The strains, judged by morphological characteristics, represented three different species. Two of the species could be identified with *Rhodospirillum fulvum* van Niel resp. *Rhodospirillum photometricum* Molisch. The third species was found to be a new one, for which the name *Rhodospirillum Molischianum* was proposed.

Regarding the isolation, culture, morphological and physiological characteristics of these organisms detailed data were given. Various organic substances can function as H-donors for these spirilla. The absorption spectra of living cells of the three species show a striking conformity, but there is a distinct difference between the absorption spectra of the brown-red *Rhodospirillum*-species and the absorption spectrum of *Rhodospirillum rubrum.* The results of a chromatographical analysis of the pigment complex of one of the strains of *Rhodospirillum photometricum* make it probable that this difference is due to the absence of the carotenoid spirilloxanthin in the brown-red spirilla.

Acknowledgments.

It is a great pleasure here to express my gratitude to Prof. Dr A. J. Kluyver at Delft and Prof. Dr J. M. W. Milatz at Utrecht for the opportunity to carry out this investigation in the Physical Laboratory at Utrecht as part of the program of the Biophysical Research Group and for the interest constantly shown during the investigation. Particularly I am indebted to Dr E. C. Wassink of the Biophysical Research Group for his great coopera-

tion and his valuable suggestions; further to Mr L. N. M. Duysens, also of the Biophysical Research Group, for his determination of the absorption spectra.

References.

1. J. Buder, Jahrb. Wiss. Botan. **58**, 525, 1919. – 2. E. Esmarch, Centralbl. f. Bakt. **1**, 225, 1887. – 3. C. S. French, J. Gen. Physiol. **23**, 483, 1940. – 4. G. Giesberger, Beiträge zur Kenntnis der Gattung *Spirillum* Ehbg. Dissert. Utrecht 1936. – 5. R. T. Hama, J. Sci. Hiroshima Univ. Ser. B, Div. 2, **1**, 157, 1933. – 6. Y. Hama, J. Sci. Hiroshima Univ. Ser. B, Div. 2, **1**, 135, 1933. – 7. A. J. Kluyver and C. B. van Niel, Centralbl. f. Bakt., II. **94**, 369, 1936. – 8. A. Manten, Phototaxis, Phototropism and Photosynthesis. Dissert. Utrecht 1947. – 9. J. M. W. Milatz, De aanslagfunctie van het metastabiele S5-niveau van neon. Dissert. Utrecht 1937. – 10. J. M. W. Milatz and N. Bloembergen, Physica **11**, 449, 1946. – 11. H. Molisch, Die Purpurbakterien nach neuen Untersuchungen. G. Fischer. Jena. 1907. – 12. J. Myers, J. Bact. **40**, 705, 1940. – 13. H. Nakamura, Acta Phytochimica **9**, 231, 1937. – 14. C. B. van Niel, Bact. Reviews **8**, 1, 1944. – 15. C. B. van Niel and J. H. C. Smith, Arch. Mikrobiol. **6**, 291, 1935. – 16. E. C. Wassink, E. Katz and H. Dorrestein, Enzymologia **7**, 113, 1939. – 17. E. C. Wassink, D. Vermeulen, G. H. Reman and E. Katz, Enzymologia **5**, 100, 1938.

(From the Department for Bacteriology and Experimental Pathology, Institute for Preventive Medicine, Leiden).

VIRUS INFECTION OF THE BONE-MARROW

by

J. D. VERLINDE

(Received June 21, 1947).

It is a subject of discussion whether a fundamental difference has to be made between microorganisms and viruses, but if it is deemed desirable to do so the classification of some pathogenic agents will give difficulties. There are transition-forms between bacteria and viruses which might be classed in the one group as well as in the other. Examples are the pleuropneumonia-like organisms, the rickettsiae and the viruses of the psittacosis-lymphogranuloma venereum group.

A virus belonging to this range was isolated from two patients admitted in the University hospital, department of children's diseases (Head: Prof. Dr E. GORTER).

I. A boy aged 13, shows attacks of fever during the last five years, at first with intervals of some months, later of 5 to 6 weeks. During the attacks the spleen and the liver are enlarged. Finally a considerable enlargement of the spleen is also observed in the intervals.

II. A girl, aged 3, is suffering from attacks of fever every two weeks for more than two years. The spleen and the liver are palpable all the time but they are enlarged more strongly during an attack of fever. Then the lymph-glands too are swollen.

Before the admission there never had been any contact between the children, they lived in quite different parts of the country.

The bacteriological routine examination, *i.e.*, aerobic and anaerobic cultures of blood and bone-marrow on blood agar plates, N.N.N.-agar and Sabouraud-agar was repeatedly negative. Once a mould was cultivated from the bone-marrow (sternal puncture) but it appeared to be a saprophyte in all probability originating from the skin. Agglutination reactions of the patients serum with typhoid and paratyphoid bacilli, *Brucella abortus* Bang and *Leptospirae*, the Weil-Felix (with Proteus X 19 en X Kingsbury), Paul-Bunnell and Bordet-Wassermann reactions were negative. In smears of the bone-marrow during an attack of fever, elementary body-like corpuscles were repeatedly found. The spherical bodies were stained well by the Giemsa and the Castaneda method and were indistinct, but anyhow Gram-negative, after the Gram-staining. They were found in the monocytes and partly extracellularly. Many of them were arranged in pairs. It was a remarkable fact that the corpuscles were not of the same size. Some of them were very small, like Paschen bodies, others were twice as large as the former.

In the periods free from fever only a very small number of uniformly sized, small corpuscles could with difficulty be detected in some monocytes.

We never succeeded in demonstrating the corpuscles in the peripheral blood, though it may be virulent as was proved by the experimental investigation.

Inoculation experiments have been carried out with 20 percent suspensions of bone-marrow in equal parts of saline and meat broth and with blood (defibrinated or mixed with liquoid ,,Roche") both taken during two different attacks of fever and in two intervals. Mice and guinea-pigs were inoculated intraperitoneally with 0.5, respectively 2 ml. Some of them died and others were killed at various times after the inoculation. The animals were autopsied under aseptic precautions and a bacteriological control of the peritoneal exudate and the spleen was made by inoculating them in glucose broth and on blood agar. Smears from the peritoneal exudate and occasionally from the spleen and the bone-marrow were stained by the Giemsa and the Castaneda method.

During two different attacks of fever, 10 mice and 2 guinea-pigs were inoculated with bone-marrow, 17 mice and 2 guinea-pigs were inoculated with blood from both patients. In the first group 6 and in the second group 5 of the mice died after 4 to 19 days. Thus the infection was lethal for 40 per cent of the mice in an average time of ten days. The other animals were killed after 10 to 14 days.

Nearly all mice, except those inoculated with blood from patient I, showed at autopsy a more or less obvious enlargement of the spleen and occasionally a slight deposal of fibrin on the spleen and the liver. In the peritoneal smears the same elementary body-like corpuscles as were present in the bone-marrow of the patients have been found. In some animals they were found with some difficulty in the spleen and the bone-marrow. In some mice killed after 10—14 days, the spleen was enlarged and the corpuscles were found in the peritoneal fluid, in others the findings were negative.

In this way, the virus could be demonstrated in the bone-marrow of both children and in the blood of patient II during two different attacks of fever. The experiments with suspensions of swollen lymph glands from patient II were negative. In feverless intervals the virus could neither be shown by mouse-inoculation nor in the bone-marrow or in the blood. This might point to the possibility either that the virus during the intervals exists in a less virulent form, or that the quantity is too low for infecting mice.

The guinea pigs killed 14 days after inoculation did not show any anomalies and elementary body-like corpuscles have not been found. The cultures of the peritoneal fluid and the spleen of all animals remained sterile.

An attempt has been made to grow the virus in the chick-embryo by inoculating 0.1 ml of a bone-marrow suspension and 0.1 ml of defibrinated blood in the allantoic cavity and in the yolk sac of 8—11 days incubated fertile eggs. The bone-marrow has been taken from patient I, the blood from patient II, both during an attack of fever. The inoculated eggs were placed at 37° C. and opened after different periods of time following inoculation. Smears of the allantoic membrane, the allantoic fluid and the yolk sac were stained by the methods of Giemsa and of Castaneda. A positive result has been obtained by inoculation of bone-marrow in the allantoic cavity. Both in the allantoic membrane and in the fluid the elementary body-like corpuscles have been found after 5 to 8 days incubation. Egg passages could be realized only by means of heavy inoculation. Suspensions of allantoic membranes in allantoic fluid proved to be virulent for mice after intraperitoneal inoculation. From 13 infected mice, 5 died after 3 to 9 days, 8 were killed after 10 days and in all peritoneal smears the elementary body-like corpuscles were found.

Enlargement of the spleen as a rule occurred 5 to 10 days after inoculation. Then the spleen proved to be virulent for mice. Mouse passages, however, could with less difficulty be realized with peritoneal washings, which may contain a mass of corpuscules.

Owing to the fact that the corpuscles in the infected mice as well as in the bone-marrow of the patients vary in size, the peritoneal fluid has been examined systematically.

Two series of ten mice were inoculated with virulent material, the one with an allantoic suspension of the first egg passage with bone-marrow from patient I, the other with defibrinated blood from patient II, both taken during an attack of fever.

The first 24 hours after inoculation the peritoneal fluid contained a number of extracellular small corpuscles. On the second day they had almost entirely disappeared whereas only a small number could be detected in some monocytes. On the third day the intracellular bodies had increased in size and resembled initial bodies. Some monocytes contained one or a few bodies, others were either teeming with them, or in the cytoplasm a large homogenous plaque like a cell-inclusion was formed.

The initial bodies as well as the inclusion-corpuscles may also be found extracellularly. By means of partial decoloration with acetone, it could be rendered probable that a cell-inclusion consists of a number of elementary bodies. On the fourth to fifth day after infection a mass of intra- and extracellular elementary bodies was found in the smears. In the following days all stages could be noted.

These findings may point to a developmental cycle of the virus in which the inclusions play an important role, possibly in accordance with Von Prowazek's theory. The variety in shape of the corpuscles in the bone-marrow depending on the phase of the disease may concur with a similar cycle in man. During the interval the virus occurs intracellularly; the infection is slumbering. When the development has proceeded and elementary bodies are getting free, fever sets in. In the latent intracellular phase the virus is but weakly virulent or not.

According to the following properties the virus appears to belong to the psittacosis-lymphogranuloma venereum group:

1. The polymorphy, the size and the staining of the corpuscles,
2. the developmental cycle,
3. the pathogenicity for mice, which might suggest a psittacosis virus of weak virulence.

By means of the complement fixation reaction no antigenic relationship with the viruses of psittacosis and of lymphogranuloma venereum could be established. The antigens used were a 5 % suspension of virulent mouse spleen and of allantoic membrane in saline. Dr Dekking kindly provided me with some convalescent sera of psittacosis and lymphogranuloma venereum.

Furthermore we failed to detect agglutinating and complement fixing antibodies in patient's sera taken off during an interval free of fever. The antigens used in the complement fixation reaction were those mentioned above. The agglutinogen was a suspension of elementary bodies obtained from washings of the peritoneal cavity of mice 5 days after inoculation and purified by centrifuging 30 minutes at 3000 r.p.m. and 1 hour at 15000 r.p.m. in the Ecco supercentrifuge.

Summary.

From the bone-marrow and the blood of two children who were suffering from a chronic disease characterized by intermittent fever and swelling of the spleen and the liver, a virus pathogenic for mice after intraperitoneal inoculation has been isolated. It could be cultivated in the chick embryo after inoculation in the allantoic cavity.

In infected mice a developmental cycle has been shown. According to its properties the virus might be one of the psittacosis-lymphogranuloma venereum group, though an antigenic relationship with the viruses of psittacosis and lymphogranuloma venereum could not be established.

(From the „Rijksinstituut voor de Volksgezondheid" at Utrecht).

A PHOTOMETER FOR THE EXTINCTIOMETRIC DETERMINATION OF THE DENSITY OF SUSPENSIONS OF MICROORGANISMS WITH MINIMAL DEVIATION FROM THE LAW OF BEER-LAMBERT

by

H. VAN GENDEREN and **R. Th. SCHOLTENS**

(Received September 22, 1947).

INTRODUCTION.

Essentially two kinds of photometers are used for the determination of the concentration of bacteria in suspensions: nephelometers and extinctiometers.

By means of the former the intensity of light, as far as it is scattered by the bacteria, is measured. The relation between this intensity and the concentration of bacteria is complicated and depends in a high degree on the minor features in the construction of the nephelometer.

The application of these instruments in bacteriology is discussed by MILATZ and ROTTIER (4).

In extinctiometers, on the other hand, the decrease of the intensity of transmitted light is measured. The procedure is applied as well for the determination of the concentration of coloured substances in solutions as for that of the density of bacterial suspensions.

According to the law of BEER-LAMBERT for coloured substances in a beam of monochromatic light the decrease of the light intensity dI in an infinitesimally thin layer $d\mathrm{l}$ is proportional to the concentration C of the substance and to the intensity of the incident light:

$$-\frac{dI}{\mathrm{dl}} = K'\ cI \qquad k' = \text{a constant.}$$

After integration we arrive at the well known equation:

$$E = \log \frac{I_0}{I_1} = Kc\mathrm{l}$$

in which I_0 is the intensity of the incident light (or in actual practice the intensity of the light after passing a cuvette with the medium), I_1 the intensity of the transmitted light; K is a constant, c the concentration of the light absorbing substance and l the thickness of the layer.

If the factor log I_0/I_1, the so-called extinction (E), is plotted in a diagram against the concentration of the coloured substance a straight line will thus be obtained.

When the coloured solution, however, is changed for a bacterial suspension scattering of light will occur and, as the intensity of the scattered light is highest in directions with small deviations from the original beam, an important part of this light will reach the fotocell (or the eye in visual instruments) together with the undeviated beam.

With increasing concentrations of bacteria the intensity of the scattered light increases (within certain limits) and that of the transmitted light decreases. Evidently instead of a straight line in the extinction-concentration diagram a curve below this line will be the result.

The degree of deviation from the law of BEER-LAMBERT depends upon the selectivity of the instrument for the transmitted, not scattered light. This lack of proportionality between extinction and bacterial density of suspensions occurs with most instruments and is a serious draw-back in the application of extinctiometry for the determination of concentrations of bacteria.

Calibration curves are in this respect of not much help, for the validity of these is restricted to the strain of microorganism used and depends upon the culture and the suspension medium [1]).

MESTRE (3) constructed an extinctiometer, based on the work of DREOSTI, in which an accurate separation of transmitted and scattered light was made by focussing the transmitted beam on the narrow opening of a screen. Only a negligible part of the scattered light passed through this opening.

The extinctions, determined for a series of dilutions of a suspension of bacteria, obeyed the law of BEER-LAMBERT over a large range of density.

It is remarkable, that this construction has found so little application. There are numerous expensive spectrophotometers available to-day, which can be made more selective in this way for the transmitted light, without appreciably greater cost.

It is the purpose of this study to give a description of another selective extinctiometer, specially designed for the use with microorganisms.

DESCRIPTION OF THE EXTINCTIOMETER.

Fig. 1. gives a diagram of the instrument. Either the cuvette C or the culture tube T are used as containers for the suspensions the density from which has to be determined. For convenience both cuvette and tube are given in the diagram.

---

[1]) In our original imperfect photometer we could not confirm the equation $E = \alpha C - \beta^2 C^2$ (α and β constants typical for the kind of suspended particles) postulated by LONGSWORTH (2) for the extinction-concentration curves of his photometer, in which the scattered light was not rigorously excluded. E/C plotted against C in our experiments did not yield as a rule a straight line.

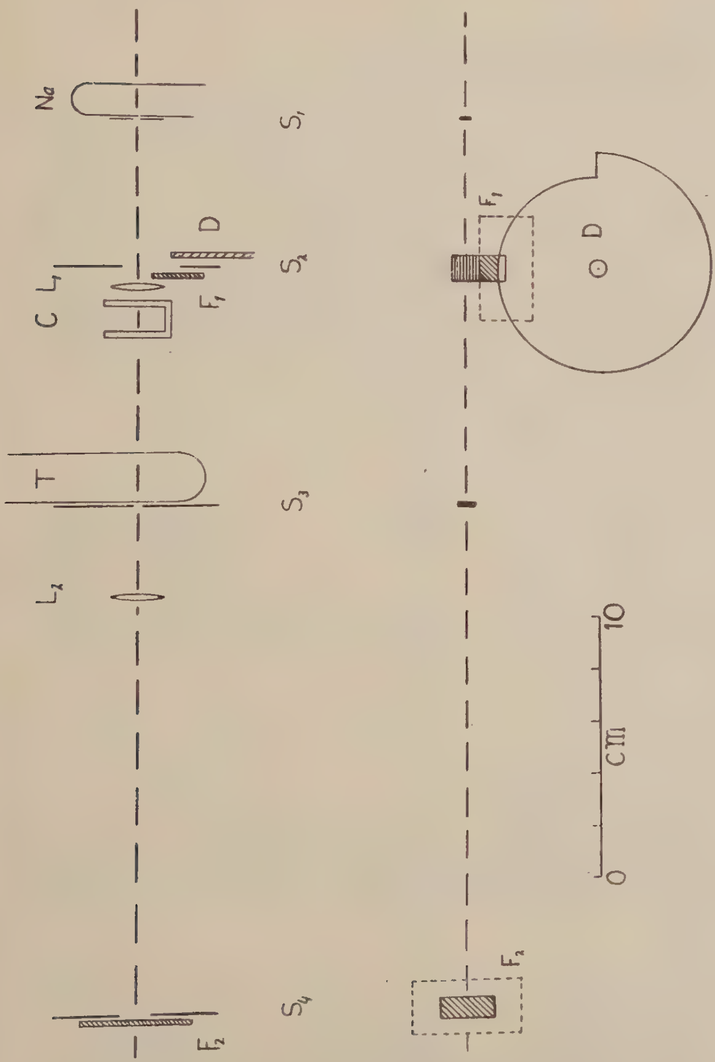


Fig. 1. Schematic diagram of extinctiometer.
Na, sodiumlamp (Philips type 4348); F_1 and F_2 barrier layer fotocells; D, spiral diaphragm; S_1, S_2, S_3 and S_4, screens, the forms and dimensions of the openings in it are given in the lower part of the figure; L_1 and L_2, both the lower lenses of 10 × Huygens eyepieces of a microscope; C, cuvette; T, culture tube.

The sodiumlamp (Na), Philips type 4348 for laboratory purpose with additional apparatus (4407) is operated on the 220 V alternative current supply. To compensate for irregularities in the voltage two fotocells are used in the well known compensation circuit of fig. 2 (For details on compensation circuits see MÜLLER (5)). The fotocells are of the barrier-layer type (photovoltaic cells) and were manufactured by Philips [1]). The position of these cells is so, that light is obtained from nearly the same spot of the light-source with the purpose of diminishing the effect of slight changes in the intensity pattern of the lamp.

---

[1]) From the agreement with the law of BEER-LAMBERT, in experiments with India ink suspensions, it can be deduced that a rectilinear relation must exist between light intensity and photocurrent in these cells under our condition of illumination.

The cuvette C is placed in the beam of fotocell F_2, the other fotocell F_1 is partly screened off by a spiral shaped diaphragm (D). By adjustment of this diaphragm compensation can be attained.

On the bulb of the sodiumlamp a screen of metal foil (S_1) is cemented with a vertical 1 × 4 mm opening on the most brilliant part, which is directed to the opening of screen S_2. Behind screen S_2 are placed the lens L_1 and the fotocell F_1. The light is focussed by the lens, after passing the cuvette, 10 mm before the narrow opening of screen S_3 and is again focussed by lens L_2 on fotocell F_2. Scattered light from the content of the cuvette will be held back by screen S_3, except for a negligible part, that will pass the aperture. Scattered light from the content of the tube T will be screened off partly by screen S_3 and most of the rest by S_4.

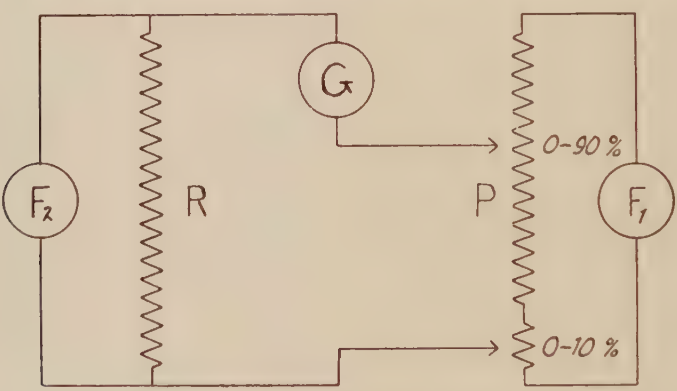


Fig. 2. Electrical circuit of extinctiometer.
F_1 and F_2, barrier layer fotocells; R_2, resistance of 300 ohm; T, double decade potentiometer of 300 ohm total resistance; G, galvanometer (Kipp, type M.R., 1 mm deflection at a metre, produced by 1.7—3.1 micro Volts or 21—13 × 10^{-9} Amperes).

In fig. 3 details of the construction are given. The frame and the supports are made of brass. The daylight is shut out by a cover made of copper with openings for the spiral diaphragm and for the insertion of the cuvette and the tube.

The handling of the instrument is simple. 15 min. before the commencement of the measuring, the lamp must be switched on. With the shutter X (fig. 3, not included in the diagram of fig. 1) closed, the zero reading of the galvanometer in the complete circuit is obtained. After insertion of the cuvette or tube with the blank medium and opening of the shutter the spiral diaphragm is adjusted to regain zero deflection of the galvanometer with the potentiometer on its full value (100 %). Then the blank medium may be changed

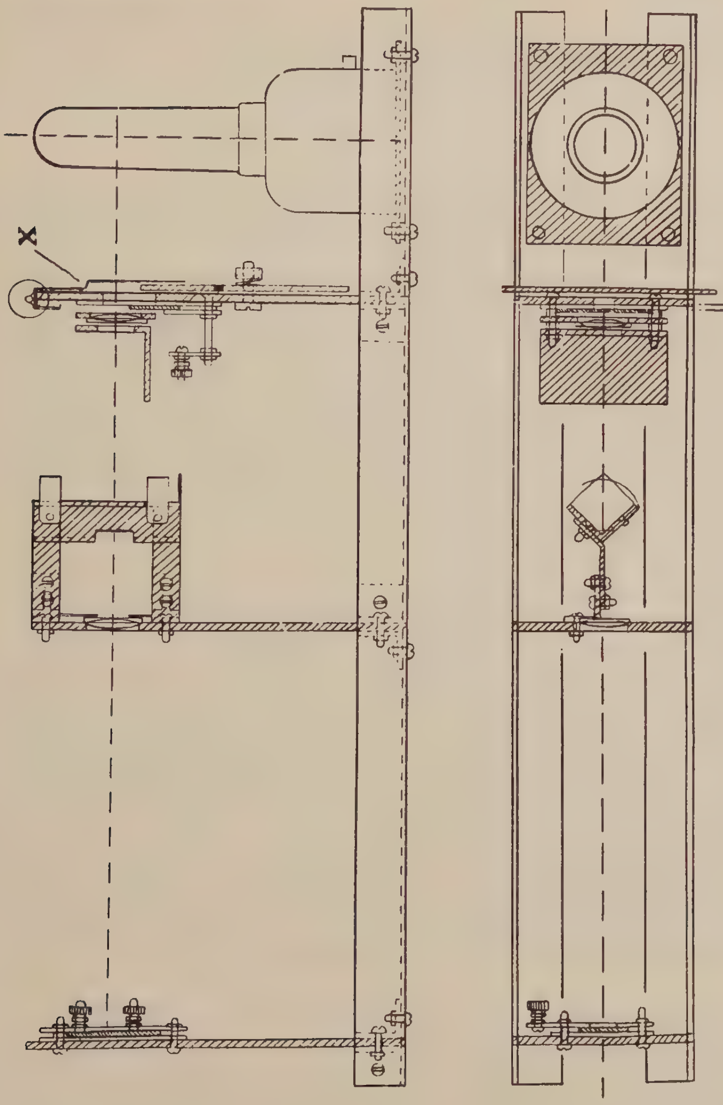


Fig. 3. Construction diagram of extinctiometer.
X, shutter, to be moved in and out of the light beam by pulling the button.

for the suspension and the potentiometer set so as to regain zero indication of the galvanometer.

The reading of the potentiometer then gives the intensity of the transmitted light through the suspension in percentage of that light going through the blank of the medium. In the equation of Beer-Lambert the extinction $E = \log \frac{I_0}{I}$ becomes thus $E = \log \frac{100}{T}$ in which T means the transmission reading of the potentiometer.

Occasionally a slight readjustment of the spiral diaphragm is necessary, probably due to a slow change in the position of the spot of maximal intensity of the sodium lamp.

DISCUSSION OF SOME FEATURES OF THE INSTRUMENT.

1. The use of monochromatic light.

MESTRE has utilised white light in his photometer and gives a table in his paper in which complete agreement with the law of BEER-LAMBERT is demonstrated for a suspension of *B. coli* over a large range of density. In our first experiments with an apparatus, based on the same optical principle with white light and with even more highly directional selectivity we could not confirm this result for different bacteria, especially not for *B.proteus*, although correct values were obtained with suspensions of baker's yeast. This difference between bacteria and yeast cells is probably due to the greater transparency of the bacterial cell. Apart from the ,,lens effect'', which has been discussed in detail by MESTRE and by LONGSWORTH the proportion of absorbed to scattered light will be greater with these bacteria than with yeast, and the influence of coloured substances in the cells (*e.g.* cytochromes) on the transmitted light will also be greater with bacteria. A suspension of *B.proteus*, seen in the direction of the transmitted beam from a white lamp is distinctly red. On these grounds it can hardly be expected that a suspension of *B.proteus* will obey the law of BEER-LAMBERT, which is only established for monochromatic light. Indeed, by the incorporation of light filters in our apparatus the results are appreciably improved, but the most positive results are gained with the light of a laboratory type sodium lamp. Sodium light, moreover, has the advantages to be the standard monochromatic light for polarimeters and other purposes; to have a low extinction in the common culture media of microorganisms, such as broth or yeast extract, and to be of rather high intensity. About 15 % of the total intensity of these lamps is made up by the red and green lines from the sodium and the neon.

2. The directional selectivity.

If the white light is replaced by monochromatic yellow light the high directional selectivity of the instrument of MESTRE proves to be not necessary to obtain results of a satisfactory quality. As the design of an instrument of this type is always a compromise between selectivity on the one hand and the cost of a more sensitive galvanometer and carefully corrected lenses on the other hand we contented ourselves with a less selective construction.

The screen aperture, essential for the separation of scattered and transmitted light in our photometer will let pass light with a maximal angular deviation from the original direction of approximately 5° vertically and approximately 2° horizontally with the restriction, that much of the light having lateral or vertical deviations between 0° and those above will also be excluded. This selectivity is about three times less than in MESTRE's photometer. Nevertheless no measurable deviation from the law of BEER-LAMBERT could be observed, as will be shown presently.

3. The use of culture tubes.

For the safe handling of pathogenic microorganisms and for some kinds of growth experiments the use of culture tubes instead of cuvettes was desired. Therefore the photometer is constructed for the use of both cuvettes and culture tubes. To make this possible the introduction of a second lens was necessary.

The position of the tube is so, that the lightbeam is focussed in the centre of the tube. The refracting effect of the tube is minimal in this position and practically no difference is noted between the image on the fotocell with or without a tube. For every tube the waylength of the light must be determined, or otherwise tubes, selected for uniform diameter must be used. The selectivity of the instrument with a tube is somewhat less than with a cuvette.

4. Errors.

The lightbeam going through the cuvette is convergent. This makes the mean waylength of the light in the cuvette about 0.1 % longer than the thickness of the liquid layer. This error is negligible, being much less than the standard deviation in the determination of concentrations. The use of a parallel light-beam would have made the photometer much more complicated.

LONGSWORTH studied the effect of orientation of bacterial rods due to circulation of the liquid in the cuvette as a consequence of heating by the lamp. This may cause errors in the determination of the extinction. In our apparatus the heat production by the lamp is so low, that never any orientation-effect could be demonstrated.

The random error will be discussed together with the results.

RESULTS.

Statistical analysis of a series of transmission readings yielded a standard deviation $\left(\pm \sqrt{\frac{\Sigma d^2}{n-1}}\right)$ in percent of the total scale-length (= 100) of ± 0.12 %. The random errors of the zero reading of the galvanometer and of the setting of the spiral diaphragm for complete compensation are included therein. The relative error of the extinction or of the concentration of bacteria (in the case of validity of the law of BEER-LAMBERT) is dependent on the transmission value, in such a way that in low and high transmissions the errors are greater than in intermediate values. The exact value can be derived from the equation (HAMILTON (1)):

$$\frac{\Delta E}{E} = \frac{\Delta C}{C} = \frac{\Delta T}{100} \times \frac{43.43}{T \log \frac{100}{T}} = \frac{\Delta T}{100} \times \frac{43.43}{TE}$$

in which $\frac{\Delta E}{E}$ is the percentage deviation of the extinction; $\frac{\Delta C}{C}$ is the relative deviation of the concentration of bacteria in percent and $\frac{\Delta T}{100}$ is the percentage deviation of the transmission.

The factor $\frac{43.43}{TE} = < 5$ for transmission values between 10 % and 75 %. The standard deviation for the corresponding extinction

Table 1.

Experiments with a 1 cm cuvette.

Dilution	Transmission readings	Mean extinction	Extinction × dilution factor	Difference from undiluted sample
	India Ink			
1 ×	15.9 % — 15.9 %	0.7986	0.7986	0.0 %
2 ×	40.0 % — 40.0 %	0.3979	0.7958	— 0.4 %
5 ×	69.4 % — 69.4 %	0.1593	0.7965	— 0.3 %
10 ×	83.3 % — 83.3 %	0.0794	0.7940	— 0.6 %
20 ×	91.2 % — 91.3 %	0.0398	0.7960	— 0.3 %
	Bacterium proteus			
1 ×	11.4 % — 11.3 %	0.945	0.945	0.0 %
2 ×	33.8 % — 33.7 %	0.472	0.944	— 0.1 %
5 ×	64.8 % — 64.7 %	0.189	0.945	0.0 %
10 ×	80.2 % — 80.3 %	0.0956	0.956	+ 1.2 %
20 ×	89.6 % — 89.6 %	0.0479	0.958	+ 1.4 %
	Staphylocccus aureus			
1 ×	15.5 % — 15.5 %	0.8097	0.8079	0.0 %
2 ×	34.9 % — 34.9 %	0.3990	0.7980	0.0 %
5 ×	69.1 % — 69.1 %	0.1605	0.8025	— 0.7 %
10 ×	83.0 % — 83.0 %	0.0890	0.8090	+ 0.1 %
20 ×	91.3 % — 91.3 %	0.0395	0.7900	— 2.2 %

Table 2.

Experiments with culture tubes.

Dilution	Transmission readings	Mean extinction	Extinction × dilution factor	Difference from undiluted sample
	Bacterium proteus			
1 ×	19.5 %	0.710	0.710	0.0 %
2.5 ×	51.6 %	0.287	0.718	+ 1.1 %
5 ×	71.6 %	0.145	0.725	+ 2.1 %
10 ×	84.7 %	0.0721	0.721	+ 1.5 %

range of 1—0.123 or concentration range of about 8—1 will be therefore $< \pm 0.12 \times 5 = < \pm 0.6$ %.

For readings between $T = 3$ % and $T = 98$ % the standard deviation in the extremes of the corresponding extinction range of 1.52—0.05 or concentration range of about 30—1 is $< \pm 0.12 \times 10 = < \pm 1.2$ %.

For $T = 1$ %—95 % the concentration range is about 100—1 and the standard deviation approximately 2.4 %.

Experimental extinctions and calculated concentrations of some suspensions are given in table 1 for cuvettes and in table 2 for culture tubes as containers. The India ink and the suspensions of washed bacteria were diluted with distilled water as given in the first column. The percentage differences from the undiluted sample (last column) indicate the composition of the error of estimation and the deviation from the law of BEER-LAMBERT. In the case of India ink these differences do not exceed expectation regarding the error of estimation alone. With the suspensions of bacteria the errors are somewhat greater. This can not be due to a specific deviation from the law of BEER-LAMBERT, caused by the influence of scattered light on the measurement, because the differences are not in one direction. Probably irregularities in the suspensions of the bacteria themselves are the cause of it. MESTRE, for this reason, stabilized his suspensions of bacteria with HCN to obtain highest precision. His errors however are of the same magnitude.

Summary.

The theory and construction of a photometer for the extinctiometric determination of the density of suspensions of bacteria are given. The application of the principle of directional selectivity and the use of sodium light leads to a satisfactory accuracy in the determination of the density of suspensions of bacteria. The results are in conformity with the law of BEER-LAMBERT. The advantages of the proposed method are discussed.

Our thanks are due to Mr. K. L. VAN LIER for his assistance in the use of the instrument.

Literature.

1. R. H. HAMILTON, Ind. Eng. Chem. Anal. Ed. **16**, 123, 1944. – 2. L. G. LONGSWORTH, J. of Bact. **32**, 307, 1936. – 3. H. MESTRE, J. of Bact. **30**, 335, 1935. – 4. J. M. W. MILATZ und P. B. ROTTIER, Zentralbl. f. Bakt. II, **94**, 227, 1936. – 5. R. H. MÜLLER, Ind Eng. Chem. Anal. Ed. **11**, 1, 1939.

(From the O.L. Vr. Gasthuis, the City Health Laboratories at Amsterdam and the State Public Health Laboratories at Utrecht).

A CASE OF ACUTE GASTRO-ENTERITIS CAUSED BY *S. MANHATTAN*

by

A. CLARENBURG, P. C. DRÖES and A. CHARLOTTE RUYS

(Received July 30, 1947).

On November 17, 1946 a 52-year old man fell ill with headache, chills and a severe watery diarrhoea. In the beginning the patient vomited several times. The faeces were brownish without blood. On the 21th he was hospitalized. He was a little dull and did not vomit any more. The tongue was dry and coated with a yellowish cover. Heart and lungs showed no abnormalities. Liver and spleen were not enlarged. Peristaltic was normal but there was a definite meteorism. The first day of admission he passed 5 times watery green stools. His temperature varied between 39° and 37° C. The urine contained a trace of albumen, the reduction was negative. There was a trace of urobilin. In the sediment some leucocytes and a few erythrocytes were seen. Blood counts gave 3.900.000 erythrocytes, 900 leucocytes. The differential count gave 26% segmented neutrophil polymorphonuclears, 56% stab-cells, 10% lymphocytes, 8% monocytes. The haemoglobin content was 90%. The sedimentation rate 34/64. The urea content was 404 mg/%, the salt content of the plasma 369 mg/%.

So the patient showed the picture of an acute gastro-entero-colitis, with uremia owing to lack of salt.

The next days as a result of an intravenous drip of water with glucose and salt the blood urea decreased, the salt content increased rapidly. The diarrhoea subsided and on 25th the stools were normal.

On 22nd the agglutination with typhoid H and O, paratyphoid A and B, *Br. abortus* and *Proteus* X 19 were negative but out of the bloodclot a *Salmonella* strain was isolated, which later proved to be *S. manhattan.*

After the patient had recovered the agglutination titer became positive and rose considerably, especially against typhoid H suspension which has an antigen in common with *S. manhattan.* The agglutinations on various dates were as follows:

	S. manhattan	*E. typhi*		*S. paratyphi* A	*S. paratyphi* B		*Br. abortus*	*Proteus* X 19
		H	O		H	O		
22/11			—	—	—	—	—	—
30/11	1 : 1000	1 : 2000	—	1 : 500	1 : 50	1 : 100	1 : 100	—
9/12		1 : 3000	—	1 : 100	—	1 : 50	1 : 50	—
13/12	1 : 500	1 : 2000	—	1 : 100	—	1 : 50	—	—
30/12		1 : 250	—	—	—	1 : 50	—	—
17/1		1 : 250	—	—	—	1 : 50	—	—

Except the first time the bloodclot never gave a positive culture. On 14.12.46 also from the faeces a strain of the same *Salmonella* was isolated. On 13.1 and 21.1.47 the cultures from the faeces were negative.

After the preliminary determination of the strain from the blood and faeces of the patient as a member of the C group, the exact diagnosis of *S. manhattan* was made in the Salmonella centre at Utrecht, which was confirmed later by Dr KAUFFMANN from Copenhagen.

As to the origin of the infection in our patient no source could be found. The patient was the only member of the family who fell ill. He worked in a milk-pasteurisation plant and his faeces had been controlled 4 to 5 months previously with negative results. One and two days before the onset of the disease he had eaten salt herring and smoked sprat. Dried eggs which might carry *Salmonella* from other countries were not eaten since the first days after the liberation because he did not like them. So there is no indication whatever as to the source of the infection.

Determination.

The organisms were motile rods, which possessed the usual morphological, tinctorial and biochemical attributes of the *Salmonella* group. Glucose, maltose, arabinose, rhamnose, trehalose, xylose, galactose, fructose, mannitol and dulcitol were fermented within 24 hours, inositol after 2 days. Lactose, saccharose, adonitol, inuline, raffinose and dextrine were not attacked. H_2S was produced but indol was not formed nor was gelatin liquefied.

Slide agglutination reactions were effected with the polyvalent *Salmonella* sera and with the Vi-serum from the International Salmonella Centre. Positive reactions were shown by the polyvalent OB- and the polyvalent HA-serum.

The cultures were agglutinated to the titre by the *S. newport* O serum (VI, VIII) and in absorption tests they removed all agglutinins from this serum. Consequently the strains contained the somatic antigens VI, VIII.

In the slide agglutination test with the different H-sera of the

C group positive reactions were only seen with the antiserum prepared from *S. typhi* (rough). So the strains contained the H-antigen d.

According to the KAUFFMANN–WHITE scheme (1940) there are 3 types in the C group (*S. münchen*, *S. oregon* and *S. manhattan*) with the same O- and specific H-antigens, but different non-specific H-antigens (resp. 1, 2 . . . .—1, 2, 3 . . . . and 1, 5 . . . .).

The cultures were examined by the swarm method according to GARD and proved to be diphasic. The non-specific phase (phase 2) was agglutinated as well by 1, 2 . . . .—1, 2, 3 . . . . as by 1, 5 . . . . serum. When tested with absorbed single factor serums [1]) this phase showed a floccular agglutination only with serum for factor 5 (a granular reaction was seen with serum for factor 3, caused by the presence of homologous O-agglutinins in this serum).

The antigenic formula of both examined strains were in conformity with that of *S. manhattan* (VI, VIII d–1, 5 . . . .).

Literature.

EDWARDS and BRUNNER (1) isolated in 1940 from a chicken a new *Salmonella*-type, which was called *S. manhattan*. The antigenic formula found by them (VI, VIII d–1, 5 . . . .) was confirmed by KAUFFMANN (2, p. 246). This strain was also found in a turkey (1) and a lizard (McNEIL and HINSHAW (3)).

SELIGMANN, SAPHRA and WASSERMANN (4) mention in their survey on salmonellosis in the U.S.A. (1939–1945) among 1107 cases of *Salmonella* infections, 7 caused by *S. manhattan*, all single cases. Out of these three were found in healthy carriers, three had predominent gastro-enteritis, one suffered from a septichaemia and one died.

References.

1. P. R. EDWARDS and D. W. BRUNNER, Amer. J. of Hygiene **34**, 121, 1941. – 2. F. KAUFFMANN, Die Bakteriologie der Salmonella-Gruppe. Kopenhagen 1941. – 3. E. McNEIL and W. R. HINSHAW, Amer. J. of Vet. Research **7**, 62, 1946. – 4. E. SELIGMANN, I. SAPHRA and M. WASSERMANN, J. of Immunology **54**, 69, 1946.

---

[1]) These serums were prepared according to the prescriptions given by KAUFFMANN (2, p. 141).

(From the Laboratory of the State Serum Institute, Rotterdam).

THE SEALING UNDER VACUUM IN AMPOULES OF FREEZE DRIED SUSPENSIONS OF BACTERIA AND VIRUSES

by

O. BOSGRA

(Received July 10, 1947).

The drying of cultures of bacteria while in the frozen state is a mode of preservation which since many years has been in use (3, 7). During the drying a definite percentage of the germs dies off, but those which survive possess a fairly unlimited term of life, provided they are stored under favorable conditions. A great advantage of this method is that the virulence, the immunological, biological and antigenic characteristics of the cultures are maintained, whereas no dissociation or mutation occurs (1, 9, 5, 11).

A virus also, when dried in the same way, can maintain its characteristics. The storing of the dried cultures has to be carried out in the absence of oxygen, thus after expelling the air. The air may be substituted by a non-oxydative gas such as hydrogen, nitrogen or carbon dioxide. It appears, however, from the literature that all those gases do not come up to a well set-up vacuum, so that the latter method is greatly to be preferred (5, 10). Also for other more practical reasons, vacuum is to be preferred to a gas filling. First of all it is not easy to administrate a filling with gas whilst any penetration of air in vial or bottle is being prevented, but the prime difficulty is, that the checking whether a gas filling is, or is not actually mixed with some air, cannot be carried out by simple means. A further drawback is, that only by laborious means it is possible to check, whether the sealing of the vials has actually prevented any leakage.

When the vials or bottles are sealed in vacuum, a thorough check on the heighth of the vacuum as well as on the presence of any leakage through very small fissures can easily be carried out by means of a high frequency test apparatus for vacuum, so that less satisfactory graded specimens may be discarded at once.

This principle of freeze drying and storing in vacuo is not only of high significance for the preservation of strains of bacteria and of viruses, but may be of great importance for the preparation of viable vaccines such as are used in veterinary therapy. Already during quite a long period research work has been carried out in the State Serum Institute in order to arrive at the production of viable vaccine in dried condition. This is only possible when these products are delivered in vials void of air.

The application of stoppers and protecting capsules on bottles could be done by means of an automatically operating capsulating machine which applies the stoppers and aluminium capsules to the matter in vacuo under high pressure (12). A drawback of the use of rubber, however, is that this material is not wholly impermeable for air and water vapour (2). A sealing by means of rubber or eventually neoprene thus can induce a limited durability of the material which correspondingly will depend on the moisture content and temperature of the surrounding air and on the quantity of the mostly very hygroscopic biological matter which is contained in the bottle. A vial, however, sealed in vacuum meets satisfactorily all that should be demanded.

Mostly vials made of pyrex glass are used for this purpose, or ordinary glass vials provided with a tube of pyrex glass fused unto them. This method serves excellently, when merely the preservation of a relatively small number of laboratory strains is concerned; it is then fairly simple to seal such vials under vacuum. When, however, a yearly production of ten thousands of ampoules is concerned, this method is very costly. Moreover these vials of pyrex glass have to be derived from foreign countries which means transit of foreign currency. All this results in the fact that the production of dried vaccine for animals can only be taken up succesfully when normal vials of ordinary glass can be used.

Moreover the possession of a sufficiently wide neck must be demanded of such vials. Research carried out to a great extent at the State Serum Institute, with the majority of viable vaccines, demands them to be freeze dried in the vial. This drying has to take place sufficiently rapidly, which demands for sufficient width of the neck. The sealing by heating of a vial of ordinary glass with a wide neck under vacuum entails, however, great difficulties. When no special precautions have been taken, the vacuum of merely half the number of the ampoules appears to be valid, whilst the sealed neck is very vulnerable. Before the expelling of the air, the neck may have been drawn out into a capillary, but even then the percentage of failures is far too high, whilst this sealed capillary is also very vulnerable. The thickness of the wall of a drawn-out neck is too narrow when compared with the width of its core.

This mode of sealing makes the proceeding very uneconomical. Thus experiments were carried out on a large scale in order to arrive at a manipulation of the vials in a manner, which would exclude failures, while rendering the sealed neck very strong.

A description may follow here of the means which will lead to such results.

As a source of heat use has been made of an interrupted ring burner (Fig. 1) with an inner diameter of 3.2 cm, which at its inner side gives rise to four small flames which in the centre of the ring burner meet at an angle of 90°. In order to render these flames firm enough, use is made of an air compressor, so that blowpipe flames arise. The supply of the gas and the pressure of the air may be regulated like in the case of a normal single blowpipe flame. This flame is used in order to make the neck of the ampoule contract circularly. Vials are used of easily melting normal Thuringia glass tubing with an outer diameter of 1.4 cm and a wall thickness of 0.9 mm [1]). By this manipulation the core of the neck is strongly narrowed, whereas the wall turns markedly thicker. For this manipulation a roundabout perfectly even heating of the neck is necessary. Would this not be the case then during the cooling down small fissures might easily occur owing to the strain occurring in the glass. Moreover the thickening of the wall on the lesser heated side would be less than on the other side and the narrowed neck would no longer be situated in the axis of the ampoule.

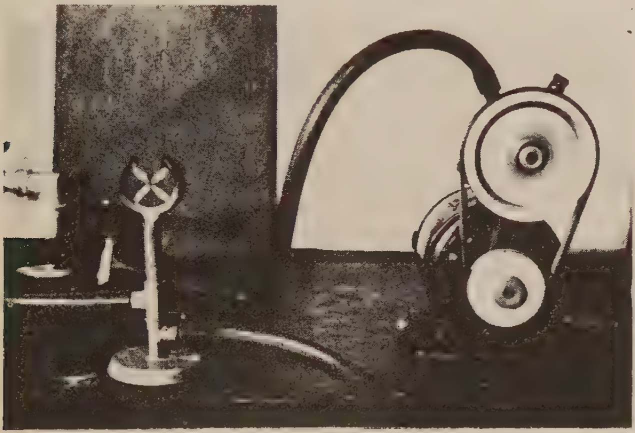


Fig. 1.

The use of the ring burner will prevent this. A slight half rotating movement of the ampoule during the circular contraction will promote still further an even heating. A great advantage of the use of the ring burner is now that a slight half rotating movement of the ampoule suffices, whilst when a single flame is used a total rotation of the ampoule is necessary. This total rotating will now and then involve a removal between the fingers, which, when the neck is hot, demands for great dexterity. When the part of the neck which has been heated, has nearly sufficiently contracted circularly, the vial is moved somewhat in the length of the vial in respect to the flame in order to get a larger part of the neck to contract and this in a direction that now the flame is situated nearer to the open end of the vial. When this is done at the right moment, the wall of the neck will contract simultaneously over its whole length. Then by means of a pedal switch, the compressor is switched off and the heated neck of the vial is removed out of the now weakly burning flames and whilst continuously rotated is drawn out somewhat. This drawing out succeeds best, when the glass having grown ,,nearly'' solid a slight pull is exerted. Moreover by these means the neck may be drawn straight, in case it might have got somewhat bended. The outer diameter of the neck will have decreased then from 7 mm down to 2 mm, whilst the inner diameter will be about 0.5 mm. The length of the treated part of the neck will be about 12 mm.

---

[1]) Vials manufactured out of tubing with either greater or smaller thickness of the wall may be treated in quite the same way.

After this treatment which actually takes but a few seconds the vial is fit for sealing. For this, after the air has been expelled from the vial, the neck is brought once more into the ring burner and slightly moved up and down. A rotating of the vial is quite superfluous. Without exerting any pull the funnelshaped end of the vial is removed whilst the vial is kept on the same level in the flame, now continuously rotating the ampoule in order to shorten the top somewhat and render it firmer. As a rule this will through its weight have deviated from the axis of the vial, and when brought in a suitable position it will be made to straighten by gravity. After this treatment the vial is quite finished.

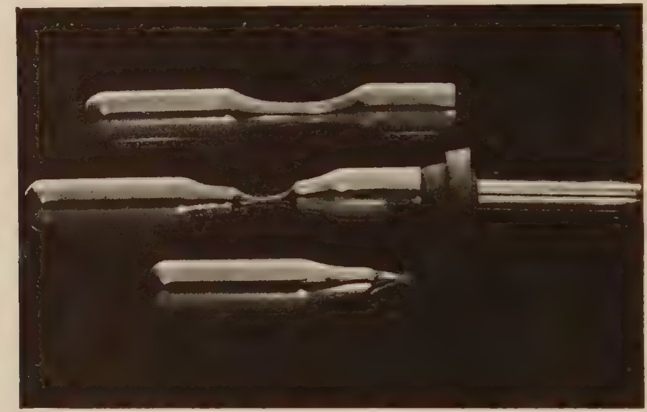


Fig. 2.

In stead of removing merely the funnelshaped part of the vial out of the flame, the vial might as well be removed by some pull. By subsequently bringing the top of the vial again in the flame this might be rendered somewhat shorter and firmer. This treatment, however, results in the occurrence of very fine fissures in the tops of about 20 % of the vials and spoiling the vacuum within 24 hours.

After some training it is possible to seal by heating without failures cheap vials of ordinary glass under vacuum (Cf. fig. 2).

Summary.

A description is given of a method for the failure-free sealing by heat of vials of ordinary glass with a wide neck under vacuum, in which viable vaccine has been freeze dried.

Literature.

1. W. J. Elser, R. A. Thomas and G. Steffen, Jour. of Immunol. **28**, 433, 1935. – 2. E. W. Flosdorp and G. W. Webster, India Rubber World **98**, 33, 1938. – 3. F. Hammer, Journ. Med. Res. **24**, 527, 1911. – 4. L. Harris, J. Inf. Dis. **8**, 47, 1911. – 5. H. E. Morton and E. J. Pulaski, J. of Bact. **35**, 163, 1938. – 6. O. Rahn, Bact. Reviews **9**, 1, 1945. – 7. B. R. Rogers, J. Inf. Dis. **14**, 100, 1914. – 8. L. F. Shackell, Amer. J. Physiol. **24**, 325, 1909. – 9. J. F. Siler, Amer. J. Publ. Health, **26**, 219, 1936. – 10. H. F. Swift, J. of Exp. med. **33**, 69, 1921. – 11. H. F. Swift, J. of Bact. **33**, 411, 1937. – 12. W. F. Verwey and S. F. Scheidy, J. Am. Vet. Med. Ass. **109**, 362, 1946.

ABSTRACTS

A. Tasman, Over het verband tusschen eiwitvoeding en immuunreacties. (On the relationship of protein nutrition to serological reactions). Chem. Weekblad **42**, 349, 1946.

Some important English and American researches on the relationship of adequate protein nutrition to the different serological reactions, are reviewed. Antibodies form part of a certain protein fraction in the serum, *viz.* the γ-globulin. This γ-globulin must be produced regularly. Part of the therefore necessary amino acids can be synthesized by the organism itself, but some have to be taken up with the food. Adequate protein nutrition thus certainly must contain these amino acids. Cannon and co-workers investigated several proteins as to their food value. They ascertained whether in rats subjected for a considerable time to diets deficient in proteins, the serum protein content and weight increased by nourishing with certain proteins. Also was shown that by feeding a low protein diet the resistance against infection diminished and recovered after sufficient feeding, whereby also the antibody formation was stimulated. F. W. B.

K. C. Winkler, Nieuwe theorieën over de vorming van antilichamen. (New theories about the formation of antibodies). Chem. Weekblad **43**, 482, 1947.

The article gives a condensed outline on the subject of antigens and antibodies with reference to the literature mainly of the last ten years.

Discussed are: the specificity of antigen and hapten, the precipitation reaction, the most important properties of the antibodies and theories about the formation of antibodies. Somewhat more amply discussed are the theory of Pauling and his method of preparing antibodies in vitro, which is criticized. F. W. B.

A. J. Kluyver, Enige belangwekkende uitkomsten, verkregen bij de toepassing van isotopen in het stofwisselingsonderzoek. (Some important results obtained by using isotopes in metabolic researches). Chem. Weekblad **43**, 164 and 180, 1947.

A review is offered of results, obtained chiefly in America during the years of the war. It is confined to researches by means of C and N isotopes, it being these which have radically changed our conception of metabolic processes. By means of C isotopes chiefly the rôle of carbon dioxide in the metabolism has been investigated. As chief result it appears that green plants, also in darkness, are capable to assimilate carbon dioxide. Heterotrophic, colourless microorganisms are, contrary to the classic view, capable to involve carbon dioxide from their surroundings in their metabolism. The same keeps true for the animal metabolism. The chemical reactions dealing with the fixing of carbon dioxide are amply discussed. As for N, the investigations concerning the N-metabolism in the animal organism are most interesting. It appears that the N, taken up as separate amino acids, is very quickly distributed among the various tissue proteins. All the experiments evidence that in the metabolism of the cell quite a complex of equilibrium reaction occur in which also the components of the cell itself are continually involved. F. W. B.

A. TASMAN en L. SMITH, De bepaling van kleine hoeveelheden ijzer in bacteriologische voedingsmedia. (Estimation of small quantities of iron in bacteriological culture media). Chem. Weekblad **43**, 248, 1947.

Small quantities of iron in the culture medium greatly affect the production of diphtheria toxin. The method here described for the estimation of iron is a modification of the method of HILL in as much that disturbance by phosphates etc. is avoided. The method consists in producing, after ashing, a complex compound of the iron and α-α'-dipyridyl, which is estimated by means of an electrical colorimeter. F. W. B.

H. L. BOOY, Aan de grens van het leven. Een inleiding tot het virusprobleem (On the border of life. An introduction to the virusproblem). Stenfert Kroese, Leiden, 1947.

A survey of our knowledge on the virusproblem is given by the author in an exceptionally thorough manner, which makes reporter regret that the book is written in a language, unintelligible to most of the scientists who should read it. That it was written in the last period of the war, when hunger, razzias, shortage of gas and coal and other troubles of the German occupation of Holland made all experimental work impossible to the author, adds to our admiration of his fortitude and perseverence and of his broad and sound knowledge of the subject treated.

The book is composed of two parts, one giving the practical aspect, the other going into theoretical considerations. Particularly attractive is the manner in which in the second part the subject is treated alternatively from the standpoint of the biologist, the chemist, the biochemist, at the end of which the author gives the synthesis of these opinions and his own view on this complicated subject. In the matter of the virus being alive or dead he joins the often given opinion that, the virus belonging to the region between living organisms and chemical molecules, the question living or dead is in a way senseless and does not disturb the biochemist, who is the explorer of this region. But this scientist is able to observe a difference in the level of life between viruses, the vaccinia virus being on a higher level than the virus of tobacco mosaic. The relation between viruses, genes and enzymes is discussed at length and therefrom a conception of the biosynthesis and the nature of viruses is derived, that offers many new viewpoints, the cancer problem included.

Professor KLUYVER (Delft) wrote a very appreciative introductory note to this volume, to which we may add our hearty congratulations to the author and the recommendation of the early issue of a translated edition. J. S.